STUDIES ON HAEMOVANADIN. III. SOME PHYSIOLOGICAL PROPERTIES OF HAEMOVANADIN, THE VANADIUM COMPOUND OF THE BLOOD OF *PHALLUSIA MAMILLATA* CUV.*

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It has been known since 1911 (Henze) that the blood of *Phallusia mamillata* Cuv. contains vanadium, which is found in special cells, called *vanadocytes* by Webb (1939). Webb estimated that the vanadocytes constitute about 60% of the blood cells and the blood cell volume amounts to between 1 and 2% of the total blood volume. It was found by Henze that the inner medium of the vanadocytes is very acid, due to free sulphuric acid which is present in a concentration of about 3%. According to Webb, the vanadium content of the animal amounts to about 0·15% of the dry weight of the body, and three-quarters of the total amount of vanadium is present in the blood, 15% in the gut and associated viscera and 10% in the mantle and branchial sac.

The pigment present in the vanadocytes is referred to as *native haemovanadin*, and the reddish brown pigment obtained following their haemolysis with distilled water as *red haemovanadin* following the terminology of Califano & Caselli (1947). On addition of alkali to a solution of red haemovanadin the pigment precipitates as *blue haemovanadin*.

The function of this vanadium compound is not known. Winterstein (1909) excluded any oxygen-carrying power (a fact which was confirmed during the present investigations). Henze (1932) found that red haemovanadin reduces methylene blue and indigo carmine, and according to Webb it reduces inorganic ferric to ferrous iron. From the reducing power Henze deduced that the vanadium should be present in the form of vanadium dioxide V_2O_2 (in which vanadium is bivalent), incorporated into a non-dialysable molecule, which he believed at first to be a protein though he later cast doubt on this opinion. Webb, on the contrary, found that the pigment is dialysable, and did not consider it to be a protein. Califano & Caselli (1947) found that the solution obtained following haemolysis contains a protein, from which the vanadium could be split by simple means, like ultra-filtration or dialysis. Califano & Caselli (1950) also determined the ultra-violet absorption curve of the haemolysate and of its dialysate.

In this paper particular attention has been paid to those properties which may be related to its physiological function. In addition we have demonstrated the presence of cytochrome *c* in sperm and in ripe eggs, and have shown that the respiration of tissues of the adult animal is cyanide-stable.

* A preliminary report of this work was presented at the First International Congress of Biochemistry, Cambridge, August 1949.

TECHNIQUES

Blood was taken from the large branchial vessel of five to ten freshly captured animals. The blood was centrifuged, the cells separated and washed either with filtered sea water or with a solution containing NaCl, KCl, KH_2PO_4 and $MgSO_4$ in the same relative proportions as for Krebs-Ringer solution (see Cohen, 1945) but corrected to a molarity of 0·6. The blood cells were then haemolysed with distilled water acidified to pH 2·4 with sulphuric acid (glass electrode). The stromata of the cells were removed by centrifugation and filtration through a glass filter, and the clear solution was then dialysed overnight at 4° C. in a cellophane sac against distilled water acidified with H_2SO_4 to pH 2·4. Dialysis removed the non-colloidal components of the haemolysate, leaving a clear solution which could be stored at 4° C. for several days without deterioration. In fact, even after a week's storage the reducing power of the solution usually decreased by only a few per cent.

The bivalent vanadium content of the solution of haemovanadin was determined, as described by Henze (1932), by methylene blue titration, using a millimolar solution brought to pH 2·4 with sulphuric acid.

Routine experiments for the detection of the spectrum of cytochrome *c* were made with a Zeiss hand spectroscope. For more accurate determinations a Beckman photoelectric quartz spectrophotometer was used.

Cytochrome *c* solutions were prepared from bovine heart by the method of Keilin & Hartree (1937), and their concentrations determined spectrophotometrically.

Catalase solutions were prepared from beef liver according to the method of Sumner & Dounce (1939).

V_2O_2 (lavender blue) was produced by reduction of ammonium metavanadate by zinc dust in the presence of sulphuric acid.

RESULTS

First of all it was found that, in order to keep the vanadium compound in the state of native haemovanadin, it is essential to maintain the pH of the medium at about pH 2·4. Therefore, haemolysis was carried out with distilled water made acid with sulphuric acid, and dialysis was also conducted against acidified water at pH 2·4. At higher pH the vanadium compound inside the cellophane sac gradually precipitates as a blue pigment. At pH 2·4 the compound does not change its colour, and the vanadium which appears in the outer solution can no longer reduce methylene blue. Thus we believe that this vanadium is no longer in the bivalent form.

The reducing power of native haemovanadin towards methylene blue seemed to offer the most promising approach for a study of its function. Native haemovanadin at pH 2·4 is only extremely slowly autoxidizable; for example, it can be stored for more than a week in the presence of air at 4° C. At higher pH the autoxidation becomes very rapid.

The addition of ferric cytochrome *c* (from beef-heart), both under aerobic and anaerobic conditions (in Thunberg tubes), at the same pH as the haemovanadin

solution (usually pH 2·4, but also up to pH 4·0) brings about a rapid oxidation of the haemovanadin together with a reduction of cytochrome *c*. The reduction of the cytochrome was followed with the spectroscope, while the oxidation of the haemovanadin was determined by titration with methylene blue. We could not follow the kinetics of the reaction spectrophotometrically, as determinations of the extinction coefficients were vitiated by a slight turbidity. Nevertheless, even by visual control it was easy to see that the reaction was rapid (it appeared complete within 2 min.) and that it was speeded up on raising the pH. For instance, the addition of a cytochrome solution of pH 3·0 to a haemovanadin solution at pH 2·4 gave quicker reduction of the cytochrome than the addition of the same amount of cytochrome which had been adjusted to pH 2·4. We assume that the haemovanadin oxidation by ferric cytochrome *c* is speeded up by hydroxyl ions.

Mention should be made of a phenomenon that occurred during our experiments. With some of our pigment preparations, both under aerobic and anaerobic conditions, the cytochrome *c* which had been reduced by haemovanadin slowly reverted to the oxidized ferric form. Under anaerobic conditions, this fact could not be attributed to autoxidation of cytochrome in the acid solution by molecular oxygen. By the addition of traces of catalase this oxidation was suppressed. We are unable to explain the phenomenon and the eventual presence of hydrogen peroxide in the reaction.

The stoichiometric ratio of the reaction was determined as follows: a standard solution of cytochrome *c* containing 0·0177 mmol. of ferric cytochrome *c* per litre was titrated with a haemovanadin solution containing 0·0025 g. atom of bivalent vanadium (estimated by methylene blue reduction) per litre. The pH of both solutions was 3·8. The addition of 0·1 ml. of the haemovanadin solution with a micropipette to 14·2 ml. of the cytochrome solution (ratio V : Fe equal to unity) gave a nearly complete reduction of cytochrome, as judged by the slight change in spectrum on subsequent addition of $Na_2S_2O_4$. The test was then repeated with a stoichiometric ratio V : Fe of one to three. The reduction was much less complete, even after 30 min. We believe that the reaction is represented by $V^{++} + Fe^{+++} = V^{+++} + Fe^{++}$ rather than by the equation $V_2O_2 + 3Fe_2O_3 = V_2O_5 + 3Fe_2O_2$, well known to chemists, and that cytochrome *c* therefore cannot oxidize vanadium beyond the trivalent stage.

We also found that a solution of V_2O_2 could reduce cytochrome *c*. The fact that haemovanadin reduces cytochrome *c* does not imply that this reduction is part of the biological function of the vanadium compound in the animal. Nevertheless, we have found cytochrome *c* in *Phallusia mamillata* Cuv. The demonstration in the sperm of the animal is quite easy if sperm suspensions in sea water are warmed to 30° C. or are treated with KCN under the spectroscope. The bands of reduced cytochrome *c* appear very clearly and resemble those seen in baker's yeast. Ripe eggs of *P. mamillata* also contain cytochrome *c*. Both in the case of sperm and of ripe eggs, we were unable to extract the cytochrome from the living cells, as the rupture of the cells causes the bands to disappear very rapidly. It is interesting to note that a similar instability of cytochrome *c* in marine animals had been noted by Ball & Meyerhof (1940).

We also tested the blood cells, branchial sac, muscles and stomach of the animal for cytochrome, but we were unable to find it.

The experiments described above are examples of interaction between haemovanadin of *Phallusia* and cytochrome *c* from beef heart. We were stimulated by a discussion that one of us (E.B.) had with Dr K. G. Paul of Stockholm to try whether the same interaction could be observed with cytochrome *c* preparations from *Phallusia*. Although so far we have been unable to isolate cytochrome *c* from *Phallusia*, we have shown that a sperm suspension will show a strong spectrum of reduced cytochrome *c* after treatment with haemovanadin.

We could find no evidence for a reaction between cyanide and haemovanadin. Several tests, giving easily reproducible results, showed that cyanide did not alter the titration value of haemovanadin against methylene blue; neither did it when haemovanadin was replaced by solutions of V_2O_2.

We wish to stress with particular emphasis the fact that we do not believe at all that the function of the vanadium compound in the blood of the animal is that of reducing cytochrome *c*, although we do believe that its physiological function is connected with its reducing properties.

SUMMARY

A method for obtaining stable solutions of haemovanadin from the blood of *Phallusia mamillata* Cuv. is described. It is essential that all operations be carried out at pH 2·4.

Native haemovanadin reduces cytochrome *c* both under aerobic and anaerobic conditions. Solutions of V_2O_2 also reduce cytochrome *c*. The reduction takes place with a stoichiometric ratio V^{++}: Fe^{+++} of one to one.

The sperm and ripe eggs of the animal contain cytochrome *c*. The pigment was not found in other tissues.

Cyanide does not inhibit the reducing power of haemovanadin towards cytochrome *c* or methylene blue.

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THE DIURNAL VARIATIONS IN THE TISSUE GLYCOGEN CONTENT AND THEIR RELATION TO MITOTIC ACTIVITY IN THE ADULT MALE MOUSE

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(With Two Text-figures)

INTRODUCTION

In a recent series of papers on the epidermis of the adult mouse (Bullough, 1949*a*, *b*, 1950*a*, *b*), it has been repeatedly stressed that the concentration of glucose or glycogen is the most important single factor affecting mitotic activity. In normal circumstances a high mitosis rate is seen only during rest or sleep, and it was suggested by Bullough (1949*a*) that this may be related to the deposition of glucose from the blood at this time. While it is well known that most of the deposited glucose is stored in the liver in the form of glycogen, it also appeared possible that some may be stored on other tissues including the epidermis.

Opportunity has now been found to check this hypothesis and to discover whether the diurnal rhythm in the glycogen content of the skin shows any relation to the diurnal rhythm in epidermal mitotic activity.

MATERIAL AND METHODS

(1) *The mice.* The animals used were males of the Kreyberg's white label and Strong's *CBA* strains, and were between the ages of 3 and 9 months. All were healthy and in breeding condition. They had been kept from birth at a controlled temperature of 20° C., and were fed on an abundance of commercial rat cake with added cod-liver oil, flaked maize, and dog biscuit. Invariably they received their food between 09.00 and 10.00 hr. so that they were accustomed to being awake at that time. This habit determined the form of their diurnal cycle of bodily activity, which in turn determined that of their diurnal cycle of mitotic activity (Bullough, 1948).

(2) *Estimation of glycogen.* Groups of animals were killed with chloroform at various times between 08.00 and 22.00 hr., and samples of skin and liver were immediately removed for glycogen determination. The method used was the Good, Kramer and Somogyi (1933) modification of Pflüger's technique. For hydrolysis, 5 or 10 ml. samples of glycogen solution, to which 5 or 10 drops of concentrated HCl were added, were maintained for $2\frac{1}{2}$ hr. in a bath of boiling water. That hydrolysis is complete in these conditions was shown by duplicate determinations on

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samples treated for longer periods, and on solutions of glycogen of known concentration.

The glucose content of the neutralized hydrolysate was determined by the Somogyi-Shaffer-Hartmann method (Somogyi, 1930), that is by the iodometric titration of reduced copper. The results were also checked by the method of Folin and Wu (Folin & Wu, 1920; Folin, 1929), which is a colorimetric technique.

(3) *Histological demonstration of glycogen.* In order to observe the distribution of glycogen in the dermis and epidermis, pieces of ear were fixed in Bouin's alcoholic fluid, embedded in ester wax, and cut into sections 7 μ thick. The sections were stained either in Best's carmine or, after treatment in periodic acid, in Schiff's reagent (McManus, 1946).

(4) *Estimation of mitotic activity.* The epidermal mitosis rate was determined in small pieces of ear removed at the time of death. All stages of mitosis were included in the counts which were made on unit lengths (1 cm.) of sections of epidermis cut 7 μ thick. From each piece of ear ten such counts were made and the average taken. Each experimental group consisted of six mice, and therefore in each case six figures were available from which a grand mean and standard error were calculated according to the method recommended by Simpson & Roe (1939) for small samples.

OBSERVATIONS

(1) *Diurnal glycogen cycles.* Judging from previous results on the diurnal variations in mitotic activity and in the blood sugar concentration (Bullough, 1949*a*), it appeared that sufficient information could be obtained from those fluctuations occurring between 08.00 and 22.00 hr. This interval includes a period of waking and feeding at about 10.00 hr., a period of sleep centred round 14.00 hr., and a period of evening activity beginning at about 17.00 hr. (Bullough, 1948).

Groups of six mice each were killed at 08.00 hr. and at 2 hr. intervals until 22.00 hr. Males were used in order to avoid both the complications of the oestrous cycle (Bullough, 1946) and the possible differences in glycogen concentration due to sex (Denel, Butts, Hallman, Murray & Blunden, 1937, 1938; Sjögren, Nordenskjöld, Holmgren & Möllerström, 1938). From each animal two samples of liver and two samples of skin were taken for glycogen determination, the skin samples including dermis, epidermis and hair. The results were expressed in mg. of glucose (formed by hydrolysis) per 100 g. of fresh tissue, averages were taken, and the results are shown in Table 1.

These results are also expressed as a graph in Fig. 1, and it is at once obvious that the glycogen content of both liver and skin reached a minimum at about 10.00 and 20.00 hr. and a maximum at 14.00 hr.

As already mentioned, the figures for the skin refer to the total glycogen content of dermis and epidermis, since it was not found practical to separate these two layers and to estimate the glycogen content of the epidermis alone. Consequently, it was necessary to check that a proportion of the glycogen measured was indeed present in the epidermis. Sections of ears taken from mice killed at 10.00 hr. and at 14.00 hr. were prepared, and were stained for glycogen. During staining slides were placed

Table 1. *The average quantities of glycogen present in the liver and skin and the average numbers of mitoses present per unit length* (1 *cm.*) *of sections* (7 μ *thick*) *of ear epidermis at various hours of the day in groups each of six adult male mice*

Time of day	Glycogen concentration expressed as mg. glucose per 100 g. of fresh tissue		No. of mitoses
	In liver	In skin	
08.00	932 ± 60	58 ± 5	4·6 ± 0·29
10.00	820 ± 84	52 ± 4	2·4 ± 0·18
12.00	1068 ± 132	68 ± 4	4·5 ± 0·15
14.00	1211 ± 87	80 ± 3	8·1 ± 0·34
16.00	968 ± 66	61 ± 4	6·3 ± 0·46
18.00	879 ± 118	58 ± 3	3·5 ± 0·21
20.00	792 ± 80	50 ± 5	1·3 ± 0·17
22.00	788 ± 14	65 ± 2	3·8 ± 0·29

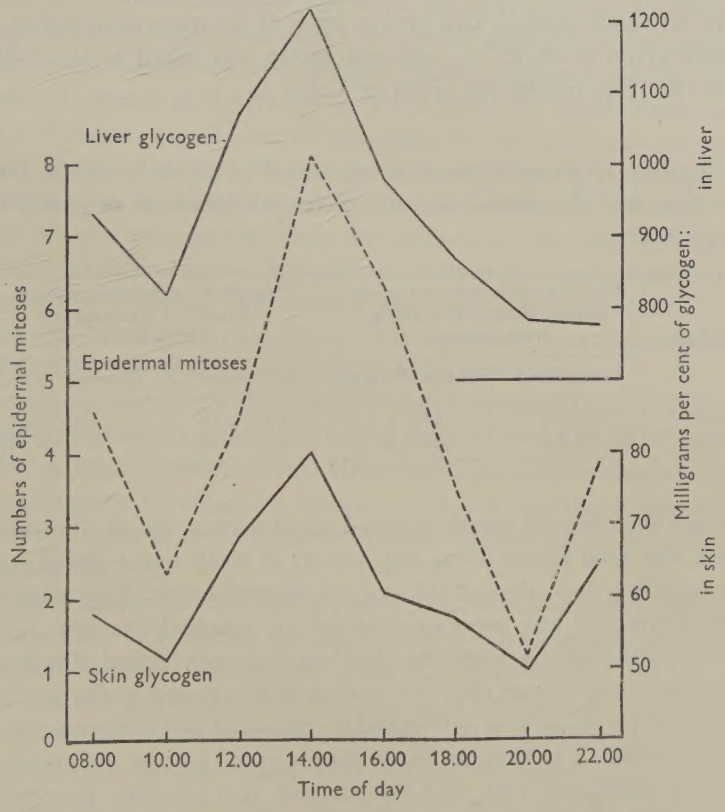


Fig. 1. Diurnal variations in the glycogen content of the liver and skin of the adult male mouse compared with diurnal variations in the epidermal mitosis rate.

back to back in pairs, one member of each pair holding sections taken from a mouse killed at 10.00 hr. and the other from a mouse killed at 14.00 hr. Comparisons between the depths of staining in such pairs confirmed that much more glycogen was present at 14.00 hr. than at 10.00 hr., and showed that at both times the greatest concentration of glycogen lay inside the epidermal cells.

(2) *Diurnal cycle in epidermal mitotic activity.* When each animal was killed a piece of ear was also removed, and from it the epidermal mitosis rate was determined. The results (see Table 1) show clearly that the diurnal variations in epidermal mitotic activity and in skin glycogen concentration ran parallel to each other. The times of high glycogen concentration were the times of high mitotic activity, and the times of low glycogen concentration were the times of low mitotic activity.

(3) *Experimental modifications of glycogen concentration.* Since glycogen concentration and epidermal mitotic activity seem normally to be linked together, it was decided to test whether this same connexion is evident in experimental conditions. To do this injections of starch or of insulin were given in the manner described by Bullough (1949*a*).

The first experiment involved the subcutaneous injection of 20 mg. starch in 0·4 ml. water into each animal, two groups each of six male mice being used. The injections were given at 08.30 hr., and one group was killed at 10.00 hr. and the other at 12.00 hr. The results are given in Table 2.

Table 2. *The effects of an injection of 20 mg. starch at 08.30 hr. on the skin glycogen concentration and the mitotic activity of the ear epidermis in groups each of six adult male mice*

Time of day	Skin glycogen concentration in mg. glucose per 100 g. fresh tissue		Numbers of mitoses per cm. length of sections cut 7 μ thick	
	Uninjected	Injected starch	Uninjected	Injected starch
10.00	52±4	58±5	2·4±0·18	3·2±0·26
12.00	68±4	94±4	4·5±0·15	7·5±0·48

Evidently the injection of starch induced an abnormal rise in the skin glycogen concentration, the final figure of 94 mg./100 g. of fresh tissue being considerably higher than anything seen during the normal diurnal cycle. This steep rise in the glycogen concentration was accompanied by an unusual increase in epidermal mitotic activity, although by 12.00 hr. this had not yet reached abnormal heights. A similar increase in glycogen concentration was also found in the liver.

The converse experiment was performed by means of two subcutaneous injections each of $\frac{1}{50}$ unit insulin. The first injection was given at 12.30 hr., when the mice were beginning their afternoon rest, and the second at 13.30 hr. The animals were killed at 14.00 hr., and the results are shown in Table 3.

The treatment induced a sharp fall in the skin glycogen concentration, and this was accompanied by a deep mitosis depression.

Table 3. *The effects of two injections, each of* $\frac{1}{50}$ *unit insulin, at* 12.30 *and* 13.30 *hr. on the skin glycogen concentration and the mitotic activity of the ear epidermis in groups each of six adult male mice*

Time of day	Skin glycogen concentration in mg. glucose per 100 g. fresh tissue		Numbers of mitoses per cm. length of sections cut 7 μ thick	
	Uninjected	Injected insulin	Uninjected	Injected insulin
14.00	80±3	54±6	8·1±0·34	1·7±0·29

Thus in experimental, as in normal, conditions the skin glycogen content and the epidermal mitotic activity fluctuated in unison.

DISCUSSION

There have been many previous studies of diurnal variations in glycogen concentration, but these seem to have referred mainly to the liver. Several species have been studied and the results obtained have been widely divergent. In 1931, Ågren, Wilander & Jorpes described cyclic variations in the liver glycogen content of rabbits, rats and mice, and found that they were to a considerable extent independent of the intake of food. Their results showed that glycogen is deposited into the liver during the night and withdrawn in the morning. As regards the rabbit these results were confirmed by Försgren (1928, 1929), who studied the deposition of glycogen in relation to the secretion of bile. He found the greatest liver glycogen concentration during the night, and the highest rate of bile secretion during the day.

As regards the guinea-pig, rhythmic fluctuations in the liver glycogen content were described by Petrén (1939), there being a minimum concentration at 09.00 hr. and a maximum at 15.00 hr. This result was challenged by Marble, Grafflin & Rachel (1940) and by Grafflin (1940*a*, *b*), who concluded that there are, in fact, no significant differences in liver glycogen content at these times.

In mice, Higgins, Berkson & Flock (1932, 1933) concluded that the diurnal cycle of liver glycogen is probably influenced by the times of feeding and of digestion, and Deane (1942*a*, *b*, 1944) confirmed this after extensive experiments with both controlled and uncontrolled feeding. A diurnal cycle was also described by Ekman & Holmgren (1949), who found maximum values during the night and minimum values during the day.

It has now been shown that the skin glycogen concentration varies with that of the liver, and from the various data available some understanding of the glycogen rhythm may be obtained. In the first place it is evident that the deposition of glycogen into the tissues occurs after feeding, as it also does after a subcutaneous injection of starch. In the second, a comparison of the present results with those described earlier by Bullough (1948) indicates that glycogen deposition occurs at the onset of a period of rest or sleep. Studies of the daily cycles of bodily activity in mice accustomed to being fed regularly between 09.00 and 10.00 hr. indicate that they have two periods of wakefulness, in the morning and evening, and two periods of rest or sleep, in the afternoon and early morning. Later results (Bullough, 1949*a*) showed

that during the periods of wakefulness the blood sugar level is high, while during rest or sleep it falls. Co-ordinating these various results it can now be concluded that at the onset of sleep glucose is deposited from the blood to form glycogen, while on waking the reverse process occurs. These relations are illustrated in Fig. 2.

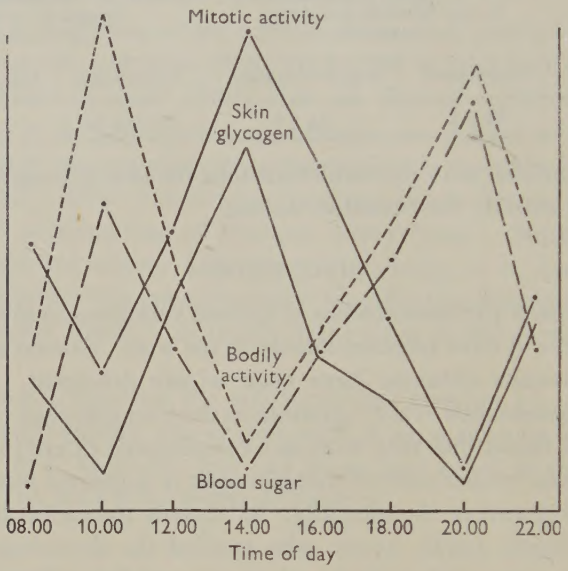


Fig. 2. The diurnal variations in the bodily activity (Bullough, 1948) and in the blood sugar concentration (Bullough, 1949*a*) compared with those in the skin glycogen concentration and in the epidermal mitosis rate.

However, it is important to stress that the timing of cycles illustrated in Fig. 2 refers only to adult male mice of between 3 and 12 months of age, which are accustomed to being fed between 09.00 and 10.00 hr. (Bullough, 1949*b*). With other age groups, with the other sex, with other species, and with other times of feeding, different forms of graphs may be expected. It is most probably the lack of standardization in these respects that is responsible for the divergent results previously reported.

The conclusions expressed graphically in Fig. 2 also lend support to the suggestion that the glucose deposited from the blood during sleep may be the critical factor causing increased mitotic activity at this time. In both normal and abnormal conditions a high concentration of glycogen in the skin coincides with a high rate of epidermal mitotic activity, while a low glycogen concentration coincides with low mitotic activity. Histologically it has also been shown that a mitotically active epidermis contains within its cells relatively large quantities of glycogen, while a mitotically inert epidermis does not. It is interesting to add, however, that it is now known that any quick depletion of tissue glycogen does not stop, or even delay, the completion of those mitoses which are at that time in progress (Bullough, 1950*a*). Apparently it is only the transition from the interphase to the prophase that is sensitive to the presence or absence of glucose or glycogen, and once a division is under way it becomes entirely insensitive.

SUMMARY

1. A description is given of the hour-to-hour variation in the liver glycogen content in adult male mice, and it is shown that the concentration is highest while the animals are asleep and lowest while they are awake.

2. A similar cycle is also described in the glycogen content of the skin. Histologically it is shown that a high proportion of the skin glycogen lies in the cytoplasm of the epidermal cells, and that during sleep both the epidermal glycogen content and the epidermal mitotis rate increase considerably. The skin glycogen content and the epidermal mitotic activity also show a marked increase after a subcutaneous injection of 20 mg. starch, while they are both abnormally depressed after two injections of $\frac{1}{50}$ unit insulin.

3. These results, together with others previously reported, are in agreement with the theory that at the onset of sleep glucose is deposited from the blood into the tissues where it appears in the form of glycogen. Since it is known that glucose, or glycogen, is a critical substance affecting mitotic activity in the adult mouse, it is logical to find that an increase in the epidermal glycogen content is accompanied by a greatly increased mitosis rate. On waking, the reverse process takes place, glycogen being withdrawn as glucose into the blood and mitotic activity falling to a low level.

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MUSCULAR AND HYDROSTATIC ACTION IN THE SEA-ANEMONE *METRIDIUM SENILE* (L.)

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(With Plate 7 and Nine Text-figures)

The majority of sea-anemones present to the eye an almost constant shape which only changes as the result of activities of a very simple kind. This is true, for instance, of *Calliactis parasitica*, the subject of an earlier study (Pantin, 1935). The unstimulated animal rarely departs from the form of a short cylinder surmounted by a disk bearing the tentacles round its edge and the mouth at its centre. In contrast with *Calliactis*, *Metridium senile* is notable for the variety of shapes which it assumes (Pl. 7). It may be fully expanded with a narrow or with a wide column (*e*, *f*). The same anemone may next day be found contracted down to a mere button-like object (*d*). It may indulge in a variety of contortions in which it is laterally bent, or possesses deep transverse constrictions locally or completely embracing the column (*g*, *i*). The whole anemone may on occasion temporarily assume a shrivelled and contracted condition (*h*), in which it might easily be discarded from the aquarium as dying; but which, in fact, is a normal temporary state from which there is comparatively rapid recovery (*i*, *j*). At other times, particularly during locomotion, a part of the body may become greatly distended (*c*). Prolonged observation of *Calliactis* shows that even here there is some variation of shape, though less evident than with *Metridium*.

Casual observation of an unstimulated *Metridium* rarely shows visible movement. But close and prolonged inspection shows that the shape of the animal at any moment is brought about by extremely slow but continual activity. We shall discuss the nature of this activity in subsequent papers. It is not simply the immediate consequence of external stimuli. But before we discuss the origin of this slow but active change of shape, it is necessary to consider the relation of the shape of the animal to the action of its peculiar system of musculature. This is the object of the present paper.

THE MUSCULAR SYSTEM

The muscular organization of *Metridium* will be discussed in another paper (Batham & Pantin, 1951). The body consists essentially of a cylindrical sac, the wall of which is composed of a layer of mesogloea, each side of which is covered with epithelium. The cavity of the sac is traversed by vertical mesenteries arising from its wall and attached to the oral and pedal disks, which close the top and bottom of the cylindrical column (Text-fig. 1). Like the body wall, the mesenteries consist of a layer of mesogloea covered on each side by epithelium.

Muscle sheets consisting of a single layer of muscle fibres are developed on one or both surfaces of the mesogloea where the epithelial layers rest upon it. The sheets consist of fields of muscle fibres which reach 1–2 mm. in length when fully extended, but which can contract down to 200–300 μ in free retraction.

This great extensibility of the muscle fibres gives the body wall very great power of deformation. Within wide limits of extension the muscles have no fixed resting length. Unlike its effect on vertebrate skeletal muscles, a light load can cause their steady extension to a new length which they retain on removal of the load with very little elastic recovery (Jordan, 1934; Kipp, 1939; the 'plastic' extension of these authors). Under natural conditions, the animal in maximal contraction becomes a flattened rugose cone in which the body wall is dense and firm. At maximal elongation, the parietal muscles of the body wall are about 400% longer than at maximal contraction (Pl. 7). The comparison of the maximum and minimum diameters of the column shows that this is also about the normal range of extensibility of the circular muscle. Isolated pieces of tissue can be extended beyond this, but very great extension is partly irreversible and ends in the tissue gently parting. In life, threads sewn into an animal for experimental purposes and subjected to the light tension of a recording lever will during many days gradually pull themselves through the extended tissue which heals up behind the wound.

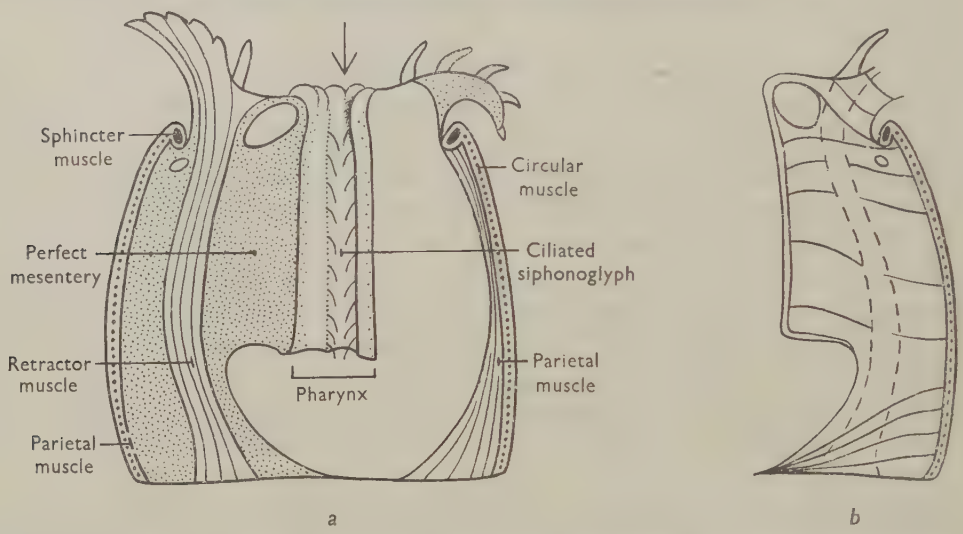
The ability of the body to undergo deformation depends not only on the extensive contractility of the muscle fibres but also on their relation to the mesogloea. We shall show elsewhere (Batham & Pantin, 1951) that contraction and extension enforces great changes in thickness of the mesogloea as a whole, but that the extensibility of that layer of it to which the muscle fibres are attached is much less than that of the body of the mesogloea. Thus the limit of extension of the column is reached when the sheet of its circular muscle is extended to form a single unfolded layer of muscle fibres. When, on the other hand, the body wall contracts and the mass of the mesogloea shortens and thickens, the circular muscle layer and the layer of mesogloea to which it is immediately attached does not shorten. It buckles; thereby throwing the circular muscle layer into folds at right angles to its fibres. When contraction approaches its limit, as in Pl. 7*d*, buckling of the muscle layer has already reached its limit and a second order of buckling, of the whole body wall, occurs. Beyond this, further shortening cannot take place. Complete unfolding and complete buckling set the limits of extension and contraction (Batham & Pantin, 1951).

This phenomenon of buckling seems to be characteristic of all the muscle sheets of *Metridium*. Where specific effectors such as the marginal sphincter or the mesenteric retractors are developed, over-growth of the muscle layer leads to the very great folding so familiar in sections of sea-anemones. Much of this folding is permanent and cannot be smoothed out during expansion of the animal but it is essentially only a special case of the buckling phenomenon.

The body wall of *Metridium* may be divided into three functional regions which are to some extent independent of each other. The region of the tentacles, capitulum and disk is primarily concerned with feeding. The region of the pedal disk is concerned with adhesion and locomotion. It is the region of the column which is

responsible for the more obvious changes of shape in the animal, and it is with this region that we are primarily concerned in this paper.

In the resting state the column is a cylinder closed at the base by the foot and at the upper surface by the oral disk. Constriction of the circular muscle fibres narrows the column. When this happens the compressed coelenteric fluid enforces an extension of the longitudinal muscles of the column system (Text-fig. 1).



Text-fig. 1. Diagram of chief musculature of column of *Metridium*. *a*, section through whole specimen, showing endocoelic faces of a non-directive perfect mesentery on left, of a young imperfect mesentery on right; *b*, exocoelic face of a non-directive perfect mesentery, showing direction of radial muscles. Broken lines indicate position of retractor muscle on opposite face.

The longitudinal musculature of the column is provided by the 'parietal' muscles. These are longitudinal bands developed most greatly in the youngest cycles of imperfect mesenteries just at the base where they are attached to the column. In the older imperfect mesenteries and in the perfect ones, strong retractor muscles become developed attaching the oral disk to the foot (Text-fig. 1); but the parietal muscle immediately in contact with the column wall is less developed. The origin and relations of the parietal system are considered elsewhere (Batham & Pantin, 1951).

The tension set up by the coelenteric pressure on the thin-walled oral disk is carried by the retractors and other longitudinal fibres of the mesenteries. In the uppermost and lowermost parts of the mesenteries these muscle fibres spread out in a fan-like manner on to the oral and pedal disks, so that they have insertions of considerable length along a radius in the two disks.

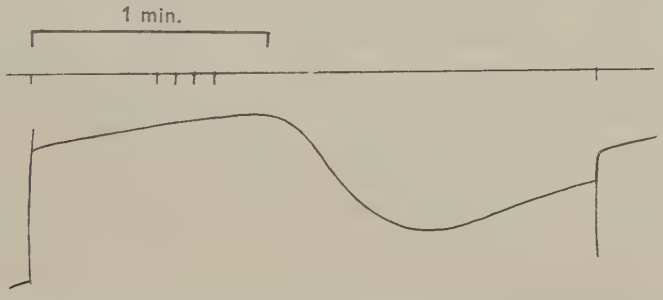
The retractors have two distinct functions. Their slow tonic contraction plays its part in maintaining the shape of the column in concert with the parietals. But on sudden stimulation their rapid contraction leads to withdrawal of the disk and closure of the anemone. In this fast response, the powerful retractors act antagonis-

tically not only to the circular muscle of the column but also to the parietals. The sudden withdrawal of the disk may cause a general bulging outwards of the whole body wall. The concurrent contraction of the marginal sphincter results in the animal passing from a cylindrical to a hemispherical shape (Pl. 7 *a*).

The only other mesenteric muscles which concern our present study are the radial muscles of the perfect mesenteries (Text-fig. 1 *b*). The contraction of these opens the pharynx. Their contraction causes vertical lines to appear at five or six places in the body wall (Pl. 7 *b*). These lines correspond to the insertion of the pairs of perfect mesenteries, the number of which varies in different individuals in *Metridium* (Parker, 1897). We shall see that this reflex is important in the control of the coelenteric volume.

THE SPEED OF MUSCULAR CONTRACTION

We will now consider the mechanics of muscular action in the column system. Muscular action in *Metridium* is extremely slow, and this important fact must constantly be borne in mind. The natural contractions of the body wall and its responses to stimulation may be extensive, but the movement accompanying these is scarcely perceptible. The parietal muscle of the body wall responds to a succession of electric shocks by contraction (Hall & Pantin, 1937). Text-fig. 2 shows such a response



Text-fig. 2. Isotonic contraction of parietal muscle of intact animal. Response to four condenser shocks at 5 sec. intervals. Contraction registered downwards.

to four shocks at 5 sec. intervals, recorded with a light isotonic lever. After the last shock there is a latent period of some 10 sec., followed by a contraction which takes some 45 sec. to reach its maximum. Such contractions are very variable, but they are always slower than those of any muscle familiar to the physiologist. The contraction recorded in Text-fig. 6 *c* took 58 sec. to develop. The subsequent relaxation was complicated, as is often the case, by further activity and was not complete till 7 min. later. This contraction was considerable, the column shortening by 2 cm. But shortening at 2 cm. a minute is not easily appreciated by the eye as movement.

The natural contractions of unstimulated animals are equally slow. The first of those in the kymograph record in Text-fig. 5 was a parietal response which took 40 sec. during the contraction phase and 4 min. for relaxation. The circular muscle of the column contracts even more slowly than does the parietal muscle (Text-fig. 9).

The extreme slowness of the contraction compels one to consider whether it is some other structure than the muscle fibres which sets up the tension. One must examine the possibility that some non-muscular system is in action; as, for instance a change of turgor in the mesogloea. We may note in passing that whatever the origin of this contraction, it can be activated by excitation of that same nerve-net, the properties of which are familiar in the more rapid retractor response (Hall & Pantin, 1937). But there is direct evidence that the muscle fibres are the contractile elements. In slices of living tissue, they can be seen to undergo contraction, whilst no other tissues, including the mesogloea, are seen to do this. Again, when a slice of tissue contracts, it always curves towards the contracting muscle layer. The mesogloea of a slice may become folded to accommodate the contraction of the muscle layer. Further, Kipp's (1939) observations on *Metridium* show mechanical parallels between the properties of rings of the circular muscle of body wall and those of other smooth muscles (Bozler, 1936). It is significant that the data given by Kipp, particularly in his figure 22, suggest that there is a hyperbolic relation between the speed of shortening of a ring of the body wall and the load against which it acts. Hill (1939*a*) has shown that this is a fundamental characteristic of the more familiar kinds of muscle. A thorough study of the load-speed relation in *Metridium* circular muscle would be of great importance in view of the fact that this stands at one extreme of the range of muscular speed in the animal kingdom.

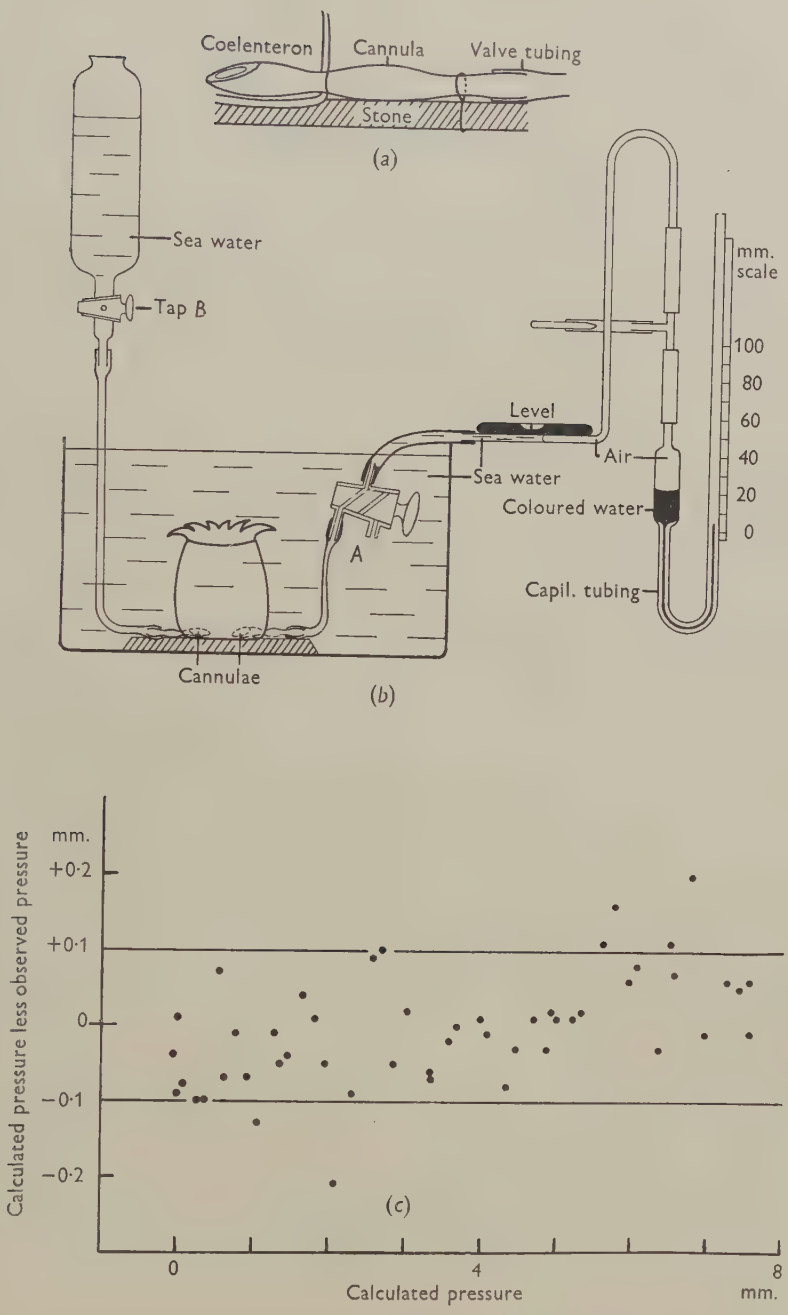
THE COELENTERIC PRESSURE AND ITS MEASUREMENT

The shape of the animal at any instant depends primarily upon the momentary state of its continual muscular activity; but the effect of this activity is controlled by two factors, the volume of the coelenteric fluid and its pressure.

Muscular activity was recorded by long, light isotonic levers lightly registering on a smoked drum. Threads attached these to the body wall of the anemone. It will be shown in a later paper (Batham & Pantin, 1950) that such treatment does not seem to affect the normal activity of the animal. Owing to the distortion of which the animal is capable, smoked drum records must be accompanied by direct visual observation if they are to be correctly interpreted.

Of various methods used to measure the pressure in the coelenteron, the following proved the most satisfactory (Text-fig. 3). A glass cannula 3–4 mm. in diameter was pushed through a small incision in the lower part of the wall of the column of an anemone temporarily anaesthetized with equal parts of $7\frac{1}{2}\%$ $MgCl_2.6H_2O$ and sea water. It is essential that the mouth of the cannula be wide to prevent blockage by the mesenteric filaments. This cannula was connected to a horizontal glass tube above the vessel containing the anemone. This tube was in turn connected to a water manometer. The wide left-hand branch of the manometer (10 mm. bore) allowed the pressure to be read directly on the 1 mm. bore right-hand branch.

The cannula side of the system was filled with water to a point about half way along the horizontal arm. To this was attached a spirit level so that it could be maintained truly horizontal. In such a system, movement of water from the animal into the tube merely causes the water-air meniscus in the horizontal arm to travel



Text-fig. 3. *a*, *b*, apparatus for measuring coelenteric pressure. *a*, detail of cannula; *b*, general view; *c*, distribution of observational error. See text, p. 268–70.

laterally without change of height. Consequently, pressure changes which cause the movement are transmitted unchanged to the air column beyond and directly registered on the manometer.

A two-way tap (*A*) enabled the connexion of the manometer system to be switched from the animal to its external sea water. The zero pressure position of the water column in the manometer was thus easily determined and its scale adjusted accordingly. For accurate pressure registration the manometer system must be chemically clean.

The position of the meniscus in the manometer was read through a powerful corrected convex lens mounted on a vertical rack and pinion movement. To avoid parallax when reading the position of the meniscus, the eye, the lens and the meniscus were alined horizontally on a distant mark at the same level. The pressures to be measured proved to be minute, and the error of observation is necessarily large compared with the smaller readings. Scale readings were judged to the nearest 0·1 mm.

To test the accuracy of the method, the manometer system was connected with a cylindrical glass vessel filled with water. By transferring small quantities of water from a burette to the vessel or vice versa, the water level could be raised or lowered. Knowing the diameter of the vessel (92 mm.) and the volume of the water added or removed, the change of pressure to which the manometer system was exposed could be exactly calculated. Accordingly, successive quantities of water were added to or subtracted from the vessel, and on each occasion the reading in the manometer was judged. To avoid observational bias, the pressures were read before the volumes.

To judge the scatter of observations, the differences between the calculated and the observed pressures were found. A zero line was then taken at the mean difference, and the individual differences plotted about this line as in Text-fig. 3*c*. Errors of observation are now evident as deviations from the mean. If observation were exact there should be no deviations and all points should fall on the zero line. If observations are correctly made to the nearest 0·1 mm., all points should fall between ±0·05 of the mean. Text-fig. 3*c* shows that the accuracy of observation falls short of this, but that about 90% of the differences fall within ±0·1 mm. of the mean. The position of the zero when set at the beginning of an experiment will be subject to this error, and so will each individual pressure reading.

When plotted over a wide range of pressures, the deviations from the mean show a slight drift from negative to positive, of about 1% of the calculated pressure. There is some indication of this in Text-fig. 3*c*. It is largely accounted for by the fact that the pressures were read directly on the capillary side of the manometer. To obtain the true value of the pressure, the slight depression of the fluid in the wide arm of the manometer should be added to the manometer reading (about 1%). If allowance is made for this, the scatter is somewhat reduced and the probable accuracy of the zero improved.

In making a long series of pressure estimates it is important to check the zero level repeatedly. Fatigue lowers accuracy of observation, and it was found least exacting to take pressure readings every 15 sec.

It is important to check the free connexion between the coelenteron and the manometer. This is easily done by slight agitation of the surface of the water containing the animal, which should cause rapid small oscillations of pressure.

From what has been said it will be evident that pressure registered by the manometer depends on the height of the horizontal connecting tube above the level of the water containing the anemone. Consequently, when registering the internal pressure of the animal relative to the surrounding medium a correction must be applied each time the water level is changed. Thus if 200 c.c. of sea water is run into the coelenteron of the anemone, the zero position must be redetermined. Apart from this correction, the apparatus correctly registers the coelenteric pressure.

CONDITIONS OF MEASUREMENT

The only remaining consideration is how far the apparatus and mode of recording might make the recorded pressure abnormal compared with that in a free-living anemone. Contraction of the animal will force some fluid into the apparatus. The coelenteric pressure does not usually exceed 10 mm. of water. This only involves the movement of 10 cu.mm. from the coelenteron into the manometer system. This small diminution of coelenteric volume may be neglected. Changes of external temperature will affect the air column between the horizontal tube and the manometer. This has no effect on the registered pressure, though a substantial rise in room temperature might force a few cu.mm. of sea water out of the apparatus into the animal. Calculation shows that the very small change of two degrees or less in room temperature during an experiment could only cause the exchange of a few cu.mm. of fluid between the apparatus and the animal.

If an animal is standing vertically with its base attached to the bottom of the aquarium, the coelenteric pressure is in equilibrium with the tension in the walls of the animal plus a small factor due to the effective weight of the immersed tissues. The anemones used in the experiments were 5–6 cm. in width and in height. The weight of an entire anemone 5 cm. broad and $5\frac{1}{2}$ cm. high from base to oral disk when suspended freely in sea water was found to be 0·76 g. Not all this weight will bear on the coelenteric fluid. With the animal attached to the substratum in the normal vertical position, the whole of the pedal disk and some of the neighbouring tissue is supported by the substratum. It is difficult to estimate how much of the anemone's immersed weight therefore is effective in helping to counterbalance the coelenteric pressure. If the whole 0·76 g. given above were borne by the fluid in an animal 5 cm. across, and if the body were completely flexible, the pressure required to support the weight alone would be $0{\cdot}76/(\pi \times 2{\cdot}5^2)$ g. per sq.cm., which is equivalent to a water pressure of 0·38 mm. This minute pressure is not negligible compared with the actual coelenteric pressure, and we find, as we would expect, that in times of extreme expansion the animal may not always be able to support its own weight and falls over to one side.

The pressure due to the immersed weight of the tissue only adds to the pressure which the coelenteric fluid must support if the animal stands vertically. If, as is

often the case in nature, it hangs downwards or sideways, the immersed weight will actually reduce, or have no effect on, the coelenteric pressure relations.

In animals in which recording levers are attached to the body wall, the tension in the recording thread will have some effect on the system. In the present experiments the tension of the thread attaching the animal to the lever is equivalent to an upward force of about 0·4 g. Acting over the surface of a disk 5–6 cm. across, this could reduce the coelenteric pressure by not more than 0·2 mm. of hydrostatic pressure. In effect, this will partly counterbalance the immersed weight of the tissue in a vertical anemone. But since the thread is attached to the more or less vertical wall of the column and not to a rigid plate in the position of the oral disk, not even the whole of this slight tension will be translated into a pressure difference.

In considering the bearing of these factors on the results, it should be borne in mind that they do not influence the correctness of the pressure values. They merely indicate the extent to which the pressure equilibrium may depart from that in a normal animal without attached threads or cannulae, standing vertically in the aquarium. The influence of the factors on the pressure equilibrium cannot exceed the changes in that equilibrium which would result if the same anemone hung vertically downwards, or extended horizontally. They cannot in any case exceed a few tenths of a mm. of water pressure.

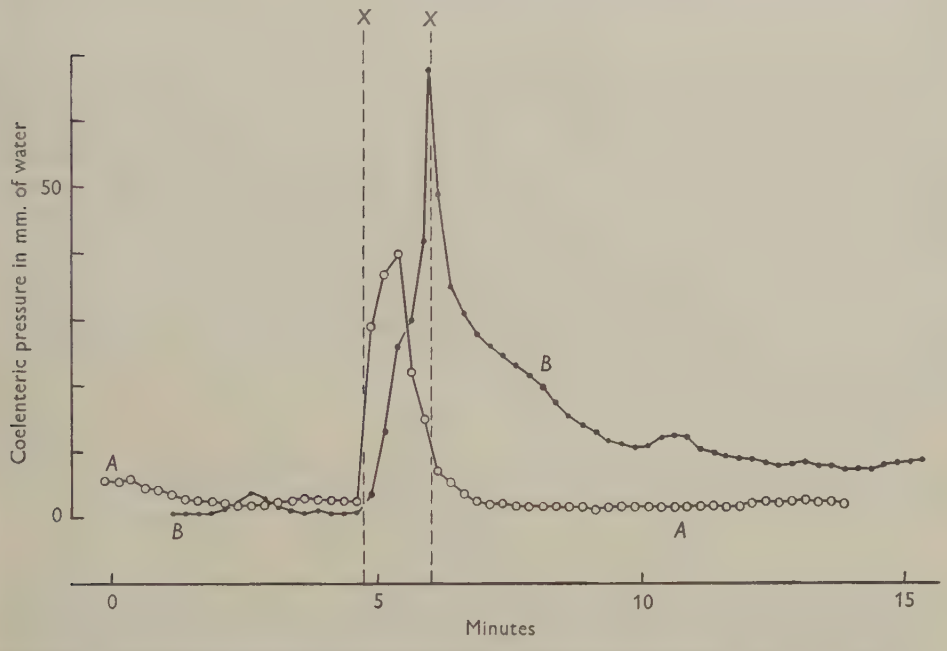
COELENTERIC VOLUME AND ITS CONTROL

A similar cannula to that of the manometer system was fixed into the opposite side of the base of the column. Through this, known volumes of sea water could be passed into the coelenteron. It proved impossible to determine accurately the volume of fluid in the coelenteron at any moment, but approximate estimates were made based upon the height and diameter of the column. A normal animal some 6 cm. in diameter at the foot and some 6 cm. from the base to the oral disk contains between 100 and 200 c.c. of sea water. The coelenteron of such an animal can be inflated to about 400 c.c. Great inflation is quite common in nature after feeding (Pl. 7*a*). The phenomenal power of anemones to expand in this way is well known. There are, however, limits to which it can usefully take place. Thus in the example given, at 400 c.c. the muscles of the body wall acting on the bloated coelenteron cannot alter its shape significantly.

Great as is the power of expansion, there seems to be a limit to which the coelenteron of an anemone can be inflated. Text-fig. 4 shows the pressure changes accompanying the rapid inflation of two anemones by 200 c.c. of sea water. In one, *A*, there is rapid loss of water owing to opening of the mouth. In the other, *B*, however, this reflex was not shown, so that leakage after filling was slow. Animal *B* was 6 cm. wide at the base and 5·5 cm. high to the mouth. The sea water was run in at a pressure of about 30–40 cm. of water. It will be seen that near the end of inflation the pressure in animal *B* starts to rise very rapidly as though a limit of extensibility is approached. We shall show that there is an upper limit to extensibility of the column when all folding of the circular muscle layer is smoothed out (Batham & Pantin, 1951).

At the other extreme, below a certain coelenteric volume the body wall becomes thick and rugose (Pl. 7 *d*). Buckling of the body wall limits the contraction of the muscle. Within these limits of inflation and deflation, however, the body may retain any volume, even when anaesthetized with isotonic $MgCl_2$ + sea water, or menthol. That is, the body behaves within the limits as a passively extensible system capable of retaining any shape imposed upon it by muscular action, without any marked tendency to elastic recoil to any particular shape or size.

The animal can control both its gain and loss of coelenteric volume. Normally, the only method of intake of water to the coelenteron is by the siphonoglyph. As Parker (1919) has said, the cilia of this beat inwards (Text-fig. 1 *a*). Its thick, firm



Text-fig. 4. Pressure changes in two specimens of unstimulated *Metridium*. Between *XX* 200 c.c. of sea water were injected into coelenteron. Animal *A* (6 cm. wide at base, 5 cm. high) showed reflex opening of mouth by contraction of radial muscles of perfect mesenteries. Note rapid fall in pressure. Animal *B*, mouth remained closed and pressure remained high.

walls provide a permanent channel for the continuous slow intake of water, though such a mechanism can apparently only produce a very low pressure. We have not yet succeeded in measuring this pressure directly in an isolated siphonoglyph. It is easy, however, to show that water can be driven down it. A glass tube 1 or 2 mm. in diameter inserted into the lower end of the siphonoglyph of an isolated actinopharynx enables one to see particles moving down it at speeds of the general order of 1 mm. per sec. But the ease with which the tube gets clogged with mucus or becomes displaced through contractile distortion of the actinopharynx presents great difficulties to the experimenter.

So long as the anemone remains constant in volume, the pressure set up by the

inflowing current of the siphonoglyph must be equal to the average pressure in the coelenteron, provided the pressure is averaged over a substantial period. The average of the average pressures during ten long experiments on unstimulated *Metridium* was 2·6 mm. of water, the individual averages varying between 0·7 and 6·1 mm. These pressures are far below the 5 to 6 cm. of pressure recorded by Parker (1917) on insertion of a glass tube into a large *Metridium*. He points out, however, that the insertion caused the animals to contract and that his pressure values were thus decidedly above normal. We shall later see that pressures of this order are obtained on maximal stimulation. But in the unstimulated animal the pressures are much lower, and we may compare them with Parker's (1919) value of 2–4 mm. of water pressure set up in the gastral cavity of the sponge *Stylotella* by its flagellate chambers.

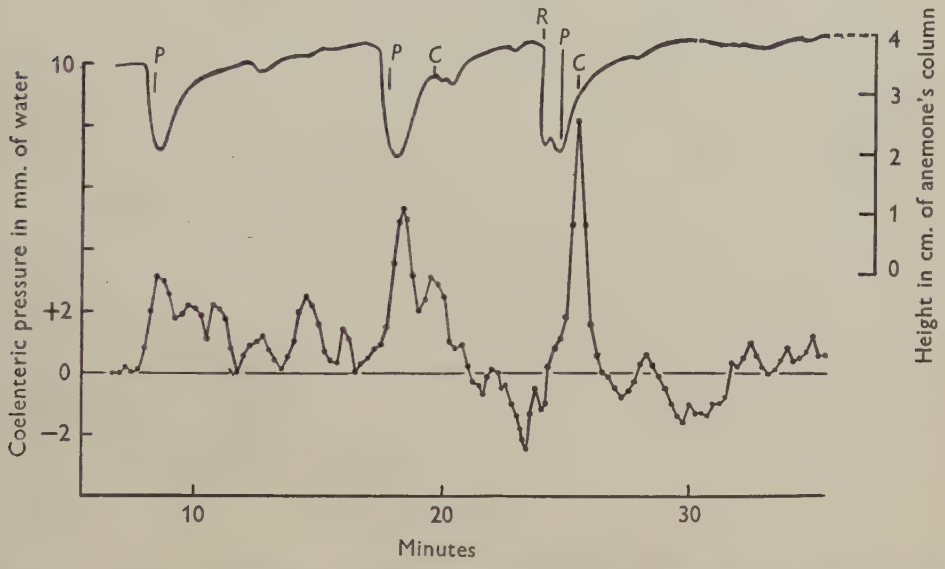
The maximum rate at which water can be taken in by the siphonoglyph in the intact animal may be hard to measure directly. But a peculiar activity of the anemone permits an indirect estimate to be made. At intervals after feeding, and in a starved animal even at other times for unassignable reasons, the whole body 'shrivels' and empties itself of water (Pl. 7*h*). It is able to refill itself in a few hours. The anemone illustrated in Pl. 7*h–j*, had been fed 48 hr. previously. At 5.20 p.m. it rapidly lost fluid and assumed the shrivelled state. Its volume, calculated from photographs, was 2·8 c.c.; 42 min. later (Pl. 7*i*) it had refilled to a volume of 6·6 c.c. whilst after 98 min. it had a volume of 20·2 c.c. The size of the recovered anemone was 2·8 cm. at the base by 4·1 cm. in height, and the rate of intake of water during the last phase was 14 c.c. per hr.

The only alternative mode of ingress of water to the coelenteron, other than by the siphonoglyph, is by actual suction. Though very small, the pressure within the coelenteron is almost always positive. The only condition under which this is not the case is in anemones which are recovering from a state of extreme contraction. Text-fig. 5 illustrates the pressure changes occurring in the coelenteron of an anemone after very severe stimulation which caused complete rugose contraction and extrusion of acontia. The record was taken when recovery had commenced and the upper part of the column was about one-third refilled. In Text-fig. 5 it will be seen that there are short periods of small negative pressure. These phases coincided probably with antiperistaltic waves which ran above the point of entrance of the cannula in the column. For such waves to produce any negative pressure at all it is necessary that the wall of the column should have some rigidity. This is in fact true during the earlier phases of recovery from extreme contraction owing to the firm character of the highly contracted body wall.

The animal is able to reduce the volume of fluid in the coelenteron. Two methods have been observed, following the introduction of excess of sea water. The more effective method is by a reflex contraction of the radial muscles of the perfect mesenteries. These open the actinopharynx, which normally acts as an effective closing valve, like a sleeve, whose walls are collapsed under even the feeble pressure of the coelenteron. Such a release of water is recorded in Text-fig. 4, animal *A*. This anemone was injected with 200 c.c. of sea water over a period of $1\frac{1}{2}$ min., marked

XX. During the first minute the coelenteric pressure rose to 40 mm. of water. It then suddenly fell even though water was still entering the anemone, and had sunk below 3 mm. within a minute of the end of the injection. During the initial stage of injection the anemone became inflated. During the fall of pressure the radial muscles pulled the mouth open and fluid escaped.

In some specimens, inflation of the coelenteron does not evoke the radial mesenteric reflex. This was the case with animal *B* in Text-fig. 4, to which reference has already been made. In such animals the pressure continues to rise throughout the period of injection of sea water and subsequently only falls slowly. During this fall, the numerous minute pores in the body wall, the cinclides, are seen to open, and fluid (and also acontia) steadily though slowly escapes.



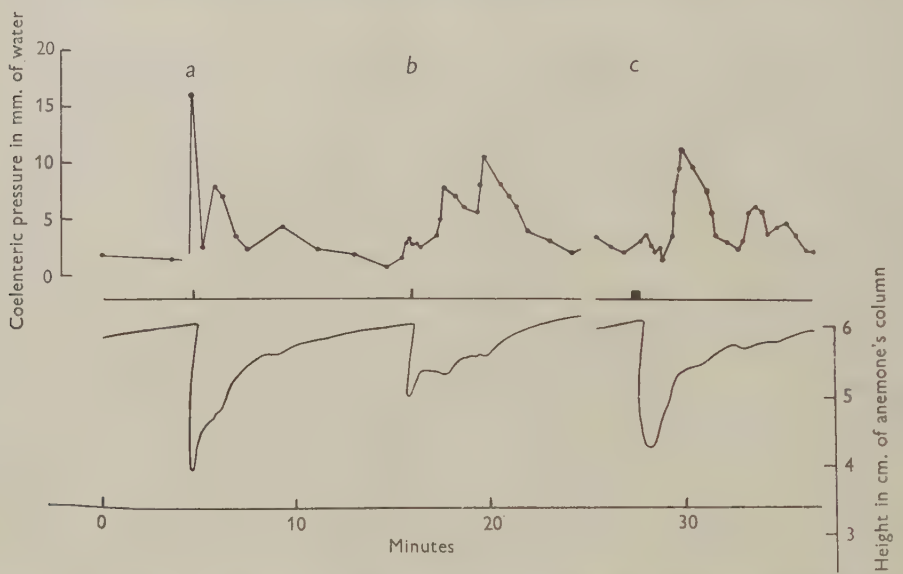
Text-fig. 5. Above, kymograph tracing of spontaneous activity, recorded with light isotonic levers. Scale on right gives approximate height of column. Below, coelenteric pressure. Note phases of small negative pressure. *P*, parietal; *C*, circular; *R*, retraction contraction.

The processes of intake and of extrusion of water occur normally during different phases of *Metridium*'s activity, so that the animal may be found to possess a very different volume at different times. But while it is in any particular phase, the animal remains for hours on end with approximately the same volume of water, and the various changes of shape which it undergoes are executed at constant volume.

PRESSURE CHANGES DURING CONTRACTION

Let us now consider the significance of pressure changes which follow activation of the muscle system of the column and the mesenteries. Hall & Pantin (1937) showed that the mesenteric retractors of *Metridium* contract rapidly in response to a series of stimuli at frequencies of about 1 per sec. Text-fig. 6*a* records the effect of a retractor response following three successive shocks at 1 sec. intervals applied to

the column. The stimuli engendered rapid and extensive contraction of the retractors which shortened the column by about 37%. This was accompanied by a sudden rise of pressure from 1·4 to 15·8 mm. This promptly fell again to 2·4 mm., after which further pressure changes occurred accompanying subsequent activity to which we shall presently refer. As might be expected, prolonged rapid stimulation produces very large and prolonged contraction of the whole musculature, with very much greater consequent rise in pressure. For a very brief response, on the other hand, while there is still considerable retraction, there is only a minute rise in pressure. Text-fig. 6*b* shows the response of the retractors to two shocks only, at 1 sec. intervals. This facilitated a single response of the retractor muscle (cf. Hall & Pantin,



Text-fig. 6. Pressure changes (above) and contraction of body wall (below) following electrical stimulation. *a*, retractor response to three condenser shocks at 1 sec. intervals. *b*, retractor response to two condenser shocks at 1 sec. intervals. Note small rise in pressure accompanying single retraction and large rises in pressure accompanying re-extension. *c*, ten shocks at frequency of 3·2 sec. elicit parietal contraction. This causes no significant rise in pressure. Subsequent re-extension accompanied by large rises in pressure.

1937), causing a contraction of about 19% in height. The pressure rose only from 1·4 to 3·2 mm.! Such remarkably small pressures are consistently obtained and are to be attributed to the diaphanous character of the body wall of an expanded *Metridium* offering little resistance to deformation. Thus, from a functional standpoint, nothing at first impeded the swift protective withdrawal of the disk by the retractors.

The retractors of *Metridium* will show fast responses to a succession of stimuli above a certain frequency; each stimulus facilitating the response of the muscle to a subsequent stimulus, provided the interval between does not exceed some 2 to 3 sec. at ordinary temperatures. Hall & Pantin (1937) found that stimulation of the column

with shocks at frequencies too low to facilitate the response of the retractors could activate the parietal muscles. They concluded that this also was a facilitation mechanism, taking place at a lower frequency than that of the retractor. Further examination, however, shows that the parietal response is more complex than can be accounted for by simple facilitation as defined by Pantin (1935). Its nature will be discussed in a later paper. At the moment we will draw attention to the fact that a battery of stimuli causing a parietal response not only initiates a contraction of this muscle, but that this is slowly followed by a complex and variable sequence of activity in the whole column musculature. Thus in Text-fig. 6*c*, ten shocks at 3·2 sec. intervals caused a substantial parietal contraction. This was accompanied by a rise of pressure from 2·2 to 3·8 mm. The pressure then fell briefly to 1·8 mm., but as the anemone re-extended itself there was a much bigger rise of pressure reaching 11·4 mm. This rise in pressure accompanying re-extension was related to a wave of contraction of the circular muscle of the column which ran downwards from the neighbourhood of the sphincter. Thus the 'relaxation' of the column after parietal contraction is an active process due to contraction of the antagonistic circular muscle layer and is accompanied by a rise in pressure; a conclusion of importance in the interpretation of mechanical records of contractions in these animals and showing how essential it is to accompany these with visual observations.

After the first wave of circular muscle contraction has passed away we see, in Text-fig. 6*c*, that subsequently a second wave follows it. Even after the brief stimuli in Text-fig. 6*a*, *b*, the initial response is followed by a succession of parietal and circular muscle activities enduring many minutes. It is not large in extent, and it is so slow that unless it is registered on a very slow-recording drum it is easily overlooked, but it shows that in addition to the immediate response to a stimulus there is lasting activity of a complex reciprocal kind between the circular and the parietal muscles.

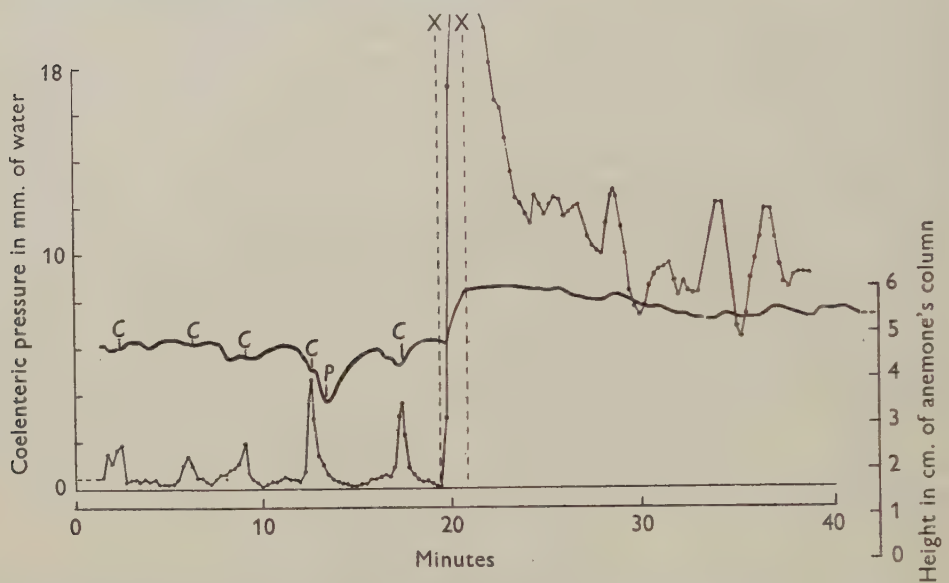
NORMAL ACTIVITY

We have remarked that the varied shapes which *Metridium* assumes are not generally caused by obvious external stimuli, but are the consequence of continual activity present in the unstimulated animal. Text-figs. 5 and 7 record the natural 'spontaneous' activity of two animals. The observed changes in pressure during this activity are also plotted. Visual observation shows that shortening is almost always due to contraction of the parietal muscles. Contraction of circular muscle generally causes elongation. But owing to distortion of the shape of the animal, local contractions of the circular muscle may sometimes have no apparent effect on the record or may even cause a fall in height of the recording point.

In the Text-figures, the incidence of parietal contractions is indicated by the letter *P*; of circular contractions by *C*; and in Text-fig. 5 is to be seen a single spontaneous contraction of the retractor muscle, indicated by *R*. There is an evident correspondence between the contractions of the circular muscle layer and rises of pressure. There may also be a rise in pressure accompanying a substantial parietal contraction (Text-fig. 5), but this is by no means always the case and, as the figures show, substantial parietal and retractor contractions may sometimes

cause little if any increase in pressure, just as we have seen in the responses of these muscles when excited electrically.

This difference in the effectiveness of circular over parietal contractions in causing a rise of pressure may be largely the result of the cylindrical shape of the animal. Since its volume is proportional to the length multiplied by the square of the radius, a small shortening of the circumference will displace a volume of fluid in the coelenteron which can only be accommodated by an enforced elongation much greater than the contraction of the circumference. To enforce this greater deformation a contraction of the circular muscle must result in a greater rise in coelenteric pressure than that following a parietal contraction of the same relative extent.

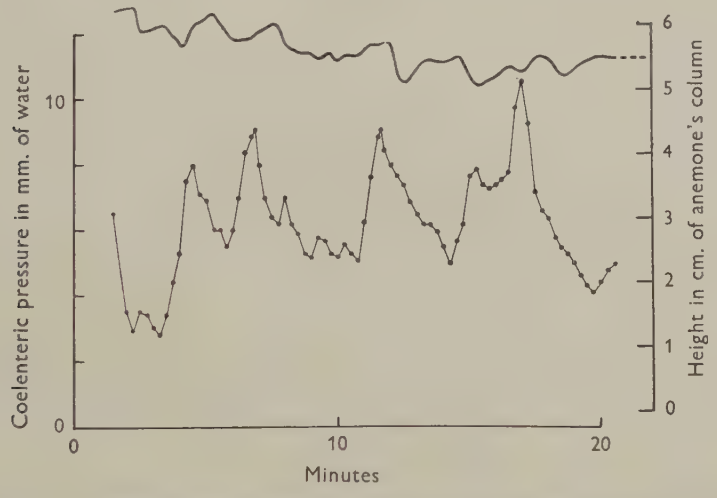


Text-fig. 7. Kymographic record of height of column and changes of coelenteric pressure, in a moderately filled animal 5·5 cm. wide and 5–6 cm. high. Light isotonic levers. Note coincidence of circular contractions *C* and rises of pressure. At *XX*, 200 c.c. of sea water were injected into coelenteron. Observe temporary obliteration of contractile activity and gradual reappearance as pressure falls. Note maximum pressure at which natural muscular activity can just cause a rise in pressure.

Text-fig. 7 records the changes in coelenteric pressure and in height of the body wall of an unstimulated *Metridium*. Before the addition of sea water at *XX* the pressures are remarkably low. In the first part of this experiment there is an average pressure of only 0·7 mm. upon which occasional rises take place up to between 1·5 and 4·6 mm.; the maxima being particularly associated with contraction of the circular muscle of the column *C*.

Animals with a low average pressure show very extensive movement. Though difficult to appreciate by eye because of their slowness, the 'spontaneous' parietal contractions occurring during the experiments shown in Text-fig. 5 reached 30–50% of the height of the animals.

Animals in this state of active movement normally have the coelenteron only moderately filled with fluid. If the coelenteron is inflated with fluid, and if this does not escape through operation of the radial mesenteric reflex, the pressure rises greatly, as we have seen in Text-fig. 4, animal *B*. The second part of Text-fig. 7 shows the effect of such inflation upon the activity. The injection at *XX* of 200 c.c. of sea water causes changes in muscle length to be almost eliminated for several minutes, only gradually to return as the average pressure value falls. The marked pressure fluctuations during this period, however, show that the muscles are by no means quiescent. Rather, they are now contracting isometrically against the increased pressure, instead of isotonically as before. At excessive volume and pressure the normal muscular activity can make little or no impression upon the shape of the animal. Inflation commonly occurs naturally particularly after a meal, and the above experiment warns us that the apparent quiescence of animals in this state may merely be the result of mechanically inefficient operation of its muscles.



Text-fig. 8. To show increased basal pressure and small changes of height in animal with great spontaneous activity.

The average coelenteric pressure is balanced by the average state of tonic contractility of the body wall. If the frequency of muscular activity increases, the average tone will increase and the average pressure must rise. This is well shown in Text-fig. 8, recorded from the same animal as that of Text-fig. 7, but on a subsequent day when it was in a much more active state. The kymograph record shows the greater frequency of contractile activity and the pressure record shows that this activity is of considerable intensity. The resulting average pressure is now 6·1 mm. of water as opposed to the 0·7 mm. maintained by this animal on a former occasion. Against this pressure, however, the muscular activity is able to execute less extensive movement; consequently, as the record shows, though activity is in fact much greater, the major changes in length, due to parietal contractions, are smaller so that the animal *seems* less active than in its original state.

These experiments go far to explain some of the major changes in shape observed in *Metridium*. When the coelenteron is well filled with sea water the shape remains remarkably constant. When it is only moderately filled, great change of shape is often evident. Nor is this all; on mechanical grounds it can be shown that the tension in the cylindrical body wall required to overcome a given coelenteric pressure will be proportional to the radius of the cylinder of the column. Hence, under those conditions in which a local contraction of the circular muscle can cause an effective constriction of the column, the smaller the diameter of the constriction the less is the internal pressure able to oppose it, and vice versa. Consequently, the shape is unstable, and a local peristaltic wave once initiated in a *Metridium* of moderate coelenteric volume and low pressure may cause great local constriction. This is amply exemplified in Pl. 7.

These results in part explain a point of some experimental importance. To perform experiments on the effects of electrical stimulation it is an advantage to choose moderately filled animals showing but little natural activity. The basal pressure of such animals is low. Initial electrical stimulation of such animals produces a very large response. But if the animal begins to show more natural activity, or if it is stimulated too frequently, the size of response elicited by stimulation rapidly falls off. Such an effect may have little to do with fatigue, but may be the result of increasing the average basal pressure against which the muscles must act.

High basal pressures such as that in Text-fig. 8 are less common than low ones. If they are maintained they must cause gradual loss of water until the pressure drops to equilibrium with the intake of water by the siphonoglyph.

MECHANICAL EFFICIENCY

The muscle system of *Metridium* is characterized by extensive free movement at very low pressures. At higher pressures, which are still small in absolute terms, the muscles cannot cause movement, as Text-fig. 7 shows. Under these conditions contraction approaches the isometric. Since this produces no displacement of the tissues it is of no direct value to the animal. On the other hand, at very low pressures, the muscle will contract almost unimpeded by any external load. The speed of contraction will now primarily depend on properties of the muscle itself. Between these extremes the muscle will shorten but will be subject to some restraint because a considerable part of its energy of contraction must be used in deformation of other parts of the body of the animal against the coelenteric pressure: that is, contraction must take place against a load which will be less than the isometric tension.

In studying the intact *Metridium* we cannot directly determine the tension developed during the natural contractions of the body wall and compare it with the isometric tension which the same muscle could develop. But the pressure in the coelenteron is a measure of the tension in the body wall, and comparison of the pressures set up during natural contractions with those set up during isometric contraction of the body wall gives us the relative load against which the muscle contracts.

We have pointed out that the average of the average pressures in ten experiments

on unstimulated *Metridium* 5–6 cm. high and 5–6 cm. wide was 2·6 mm. of water. The maximum pressures accompanying natural contractions during these experiments ranged from 3·6 to 10·8 mm. with an average maximum pressure of 6·5 mm. of water.

We can estimate approximately the pressure in equilibrium with isometric contraction in these animals in two ways. We can examine the pressure records of anemones distended with sea water and note at what pressure the natural contractions of the body wall can just cause the pressure to increase. In Text-fig. 7 such rises of pressure can be seen to reach 12·6 mm. Seven observations on the series of anemones under discussion gave values ranging from 10·1 to 12·6 mm. with an average of 11·2 mm. Since, however, these natural contractions are local in the body wall, they are likely to be accompanied by some slight extension of the rest of the body. They are therefore not completely isometric and the value of 11·2 mm. is probably somewhat too low.

An alternative method of estimating the isometric pressure is to cause a maximal contraction of the parietal and circular muscle system by prolonged electrical stimulation by shocks at frequencies of the order of 1 every 3–10 sec. When this is done, the pressure rises. It may do so in several steps and estimates of the isometric pressure by this method must be regarded as very approximate. However, the first maxima in ten experiments of this kind ranged from 12·5 to 20 mm. of water with a mean at 16·1 mm. Further stimulation gave irregular and much higher values, ranging from 12·5 to 50 mm. Under such conditions the shape of the animal becomes greatly deformed and the numerous mesenteries contract powerfully so that the pressure values may be far above that which the body wall itself can sustain in its normal cylindrical shape.

In the light of these two kinds of experiment it seems that for *Metridium* 5–6 cm. high and 5–6 cm. wide the isometric pressure which could be developed lies between 11 and 16 mm. of water.

The natural contractions of *Metridium* vary greatly in size, and the pressures accompanying them range from the average basal pressure of 2·6 mm. up to the average maximum of 6·5 mm. That is, the average pressures during natural contractions range from about 20 to 50% of the isometric pressure. These figures are of some interest. Fenn's (1923) work on the frog's sartorius showed that the amount of external work done by a single contraction showed a broad maximum for loads round about 40% of the isometric tension. Hill (1939*b*) gives the load for maximum mechanical efficiency and that for maximum power as 46 and 31% respectively for frog skeletal muscle. If the mechanical properties of *Metridium* muscles resemble those of *Rana* it would follow that the natural contractions of *Metridium* took place over the optimal region of mechanical efficiency. Such a resemblance between two muscle systems whose speeds differ by a factor of about a thousand (Text-fig. 2) would indeed be remarkable, though ever since the work of Hill (1926) and of Levin & Wyman (1927) an essential similarity has been evident in muscles of diverse kind and from animals from almost every major phylum (Ritchie, 1928).

THE TENSION IN *METRIDIUM* MUSCLE

Though the coelenteric pressures of *Metridium* are small, the tension of the body wall required to produce them is not. For mechanical reasons, it is convenient to estimate this with respect to the layer of circular muscle fibres of the column wall. The parietal system which antagonizes this is more complex since some of the pressure on the disk and foot is met by mesenteries, as we have noted. The circular muscle of the column, being at right angles to the parietal and mesenteric system, can mechanically be treated separately from it so long as the animal remains approximately a cylinder.

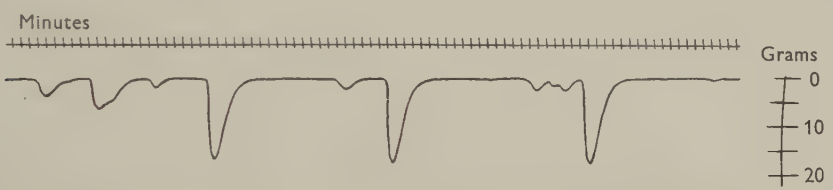
For a cylinder of infinite length the tension per cm. in its wall across a line parallel to its axis is equal to the internal pressure multiplied by the radius of the cylinder. In the anemone the cylinder is not of infinite length, and is closed by two 'plates', the oral disk and the foot. In the immediate neighbourhood of these 'plates' the situation is more complex, but over the greater part of the cylinder the relation will hold.

The anemone considered in Text-fig. 7 was 5·5 cm. in diameter. At the average basal pressure of 0·7 mm. water in the first part of the record the tension will thus be only 0·19 g./cm. of the body wall. But the maximum natural contraction reaches 4·6 mm. of pressure, corresponding to a tension of 1·3 g. After inflation with sea water the maximal natural contraction which causes a rise of pressure reaches 12·6 mm., corresponding to a tension of 3·5 g./cm. of body wall. We have seen that the isometric tension may slightly exceed this.

We can check this value for the isometric tension by another method. The maximum isometric tension developed by a ring of known width from the body-wall tissue can be determined directly. This method has the advantage of directness. It has also the advantage that all tension is unequivocally the result of contraction of the circular muscle; the primary mesenteries can be removed in preparing the ring of tissue, and all connexions with the oral and pedal disks are necessarily severed. It has the disadvantage that cutting a ring of body wall at first causes great contracture, so that it is rather difficult to estimate the width it occupied in the intact column, or to prepare such a ring with a constant width.

The following method gave satisfactory preparations. A *Metridium* of known dimensions was allowed to expand and then anaesthetized for 2–4 hr. in $\frac{1}{2}MgCl_2 6H_2O + \frac{1}{2}$ sea water. The animal, still expanded, was placed on its side and a circular cut made 1 cm. below the junction of the scapus and capitulum. The upper part thus severed, including the marginal sphincter, was discarded. A second cut was made equatorially round the column, and a third 1 cm. above the pedal edge; the pedal disk being discarded. Two rings of tissue were thus prepared of approximately equal width. These rings were then transferred to sea water for at least 24 hr. and stretched by a light load. They were then suspended in sea water and their tensions recorded isometrically. It is not always easy to get maximal contractions to electrical stimuli in these circular muscle preparations. They respond well, however, to prolonged gentle tactile stimulation. Moreover, as time passes after their first preparation, spontaneous contractions appear which finally become maximal and of constant size.

Text-fig. 9 records the maximal contractions of a ring of body wall occupying 2 cm. of width in the original expanded animal. The animal in this case was 5 cm. across the pedal disk and 6 cm. in height. It will be seen that an isometric tension of 18 g. was developed. Since the width of the ring was originally about 2 cm. in the expanded column, the total width of tissue in the two sides of the ring is 4 cm. Thus the isometric tension is about 4·5 g./cm. of the expanded column. Experiments such as this yielded values from about 2·5 to 4·5 g./cm. of column. These values are consistent with those obtained by Kipp (1939) by stimulation of ring preparations of the body wall of *Metridium*. His preparations could lift a load of at least 15 g. In a private letter he kindly tells us that the width of the rings, in the expanded animal, was about 3 cm. The isometric tension was therefore somewhat in excess of 2·5 g./cm. of column. It is evident that values of 2·5 to 4·5 g./cm. of the expanded column measured directly on ring preparations are fully consistent with the value of 3·5 g./cm. deduced from the pressure measurements.



Text-fig. 9. Isometric kymograph tracing of spontaneous activity of circular ring of column wall, Each horizontal scale division equals 1 min. Vertical scale at right indicates amount of stretching of ring caused by known weights in grams.

Perhaps the most remarkable feature of this figure is the very high value it seems to imply for the tension in the individual muscle fibres. The circular muscle sheet of *Metridium* consists of a single layer of muscle fibres. We shall show elsewhere (Batham & Pantin, 1951) that for *Metridium* of the range of size used in these experiments the number of fibres per cm. of body wall in a fully expanded animal is about 10,000 and is fairly constant. Contraction causes the muscle layer to buckle and when this buckling is extreme the number of muscle fibres per cm. of body wall is about 35,000. Animals in a state of expansion comparable to the inflated specimens on which the isometric pressure was determined will not have more than some 20,000 fibres/cm. Owing to their excessive thinness, the diameter of the muscle fibres in extreme expansion cannot be measured accurately. Our approximate measurement of the diameter was 0·7 μ. The fibres are arranged one for each micron of the muscle layer and there is a space between each extended fibre of at least 30% of their distance apart. Twenty thousand fibres of 0·7 μ in diameter have a total cross-section of nearly 8×10^{-5} sq.cm. If the body wall can exert 3·5 g./cm., the fibres themselves must exert a tension of rather more than 40 kg./sq.cm. of their individual cross-section. Such an estimate is necessarily only approximate. It involves the square of the diameter of the muscle fibres, and this cannot be accurately determined. But it is significantly greater than that for frog's skeletal muscle. Ramsey & Street (1940) found that a single fibre, apparently of the semi-tendonosus,

developed a tension of 3·5 kg./sq.cm. of cross-section of the fibre. To carry a tension of 3·5 g./cm. in the column of *Metridium*, muscle of this kind would have to occupy a solid layer 10μ thick, apart from any supporting structure. No such equivalent thickness of muscle is to be found even when every allowance is to be made for folding of the muscle layer: the circular fibres would have to stand fourteen deep.

Though improved methods of measurement are needed, it seems that the tension in the fibres of the circular muscle of the body wall of *Metridium* is large compared with that in those of a frog's skeletal muscle. Though many workers have studied many kinds of muscle they have rarely recorded the size of the muscles on which they experimented; consequently data relating isometric tension to cross-section seems to be rare. Bozler (1930) mentions that the columella muscle of *Helix pomatia* can exert 5 kg./sq.cm., and Marceau (1909) found that the 'slow' component of lamellibranch adductors gave very high values, reaching 35 kg./sq.cm. in *Venus*. Here, too, it is very slow muscles which give so large a tension. It is noteworthy that high as are these values they are still far below the breaking strain of tendon. Meyer (1942) quotes a value of 1100 kg./sq.cm. for the breaking strain of tendon of a pig's foot.

MUSCULAR ACTION AND THE HYDROSTATIC SKELETON

We have shown that muscular action of the column in *Metridium* takes place against a coelenteric pressure ranging from a basal average pressure of about 2½ mm. up to an average maximum of about 6½ mm. of water, and we have shown that this probably corresponds to a range of from about 20 to 50% of the isometric pressure. Whether this range corresponds here as in the muscles of other animals to the region of maximum mechanical efficiency remains to be seen. From a functional point of view, however, mechanical efficiency is only of indirect importance to the animal, particularly in animals such as *Metridium* whose movements are so slow that virtually no external work is transformed into kinetic energy. For such animals, the overriding objective of any movement is to get a particular part of the animal into a particular position whatever the external work that must be done. A tentacle must carry a piece of food to the mouth whether it is light or heavy: there is no profit in a mechanically efficient contraction which carries the food only half way. What might be called the 'functional efficiency' in such cases would be greatest when a given expenditure of energy in the muscle produces a maximum displacement of tissue, rather than a maximum of external work. Experiments such as we have described leave no doubt that the animal is able to execute the largest natural movements of the column over the range of coelenteric pressures mentioned above. Most of the fantastic variation of shape shown in Pl. 7 is executed in this range of pressure.

If the pressure is too near the isometric, contraction cannot cause large displacement of tissue. Above about 12 mm. pressure, the circular-parietal system of the column is unable to cause any change of shape. But there is one reaction of *Metridium* which involves rapid and forcible distortion of shape which may necessitate a much greater rise of coelenteric pressure. While, as we have seen (Text-fig. 6), a

single response of the mesenteric retractors may cause a remarkably small concomitant rise in pressure, the rapid completion of this vital protective reaction involves pressures far above 12 mm. We found that prolonged stimulation of the anemones used in these experiments by shocks at a frequency of 1 per sec. activated the retractor muscles and caused complete retraction accompanied by pressures ranging from 40 to 100 mm. of water. Movement against such coelenteric pressures can only be executed by relatively powerful muscles: the 'isometric ceiling' must far exceed that of the circular-parietal system. This is, of course, the functional explanation of the well-developed retractor muscles in *Metridium*, where by repeated folding of the muscle sheet a considerable tension per sq.cm. of the disk can be exerted. If it were not functionally essential that retraction should be rapid the tissue displacements involved would only necessitate pressures of a much lower order, and the mesenteric retractors might be less strongly developed; as is, in fact, the case in *Calliactis*. If the distortion is even more extensive than in *Metridium* and must take place even more rapidly, the retractors must be even more developed. This appears to be the case in burrowing anemones like *Peachia* with their extremely powerful retractors. The Ceriantharia, with their highly folded ectodermal muscle sheet, seem to have solved the same problem by a different route.

In animals with a hydrostatic skeleton, the 'isometric ceiling' for their various movements must be related to the work which normally has to be done. In *Metridium*, even for active retraction, this ceiling does not have to be very great when measured in absolute terms. In burrowing animals much more work must be done to execute movement in the natural environment, and the isometric ceiling must be higher. Chapman & Newell (1947) have given an interesting discussion of the role of the body fluid in *Arenicola*. In this case the hydrostatic skeleton is provided by the coelomic fluid. As in *Metridium*, rise in pressure is particularly related to circular contraction but, as might be expected, the internal pressures in this burrowing animal are much higher than those in *Metridium*. They give a 'resting' pressure of about 130 mm. of water, whilst the maximum pressures in active worms reach some 300–360 mm. These authors have been kind enough to inform us that the maximal pressures developed in the coelom of *Arenicola* on maximal stimulation are of the order of 800–900 mm. of water. It thus seems that in this case, also, the working range of the body-wall contraction is in the region below 50% of the isometric pressure, as in *Metridium*, though at a much higher level of pressure.

In animals which, like *Metridium*, possess a hydrostatic skeleton, the high pressure and deformation following maximal retraction for a time disorganize the whole mechanism of response in a way which is without parallel in animals with jointed skeletons, like the Vertebrates. This is due to the peculiar nature of muscular action where the skeleton is a mass of fluid, or a highly plastic parenchyma, invested by a muscular sheet. Such a system is found in many phyla and indeed in the viscera of almost all animals. Mechanically it is very different from the skeletal system of animals with a hard skeleton like the Vertebrates and Arthropods. In these, displacement of parts is effected through levers and substantial movements are accompanied by only small changes in muscular length. In the hydrostatic skeletal system

muscular contraction involves far greater change in length. The contraction of the muscle may be of almost identical extent with the movement of a part, as in the carriage of the prey to the mouth by the contraction of an extended tentacle of *Hydra*. Such extensive movements cannot be performed without great deformation, and if they are performed quickly there must be great waste of energy in this. The slowness of muscular action in *Metridium* has been repeatedly illustrated, and in general the hydrostatic skeletal system is characteristic of slow-moving animals. Only in such slow animals can great tissue deformation be executed without great inefficiency.

It is a prime feature of the hydrostatic skeletal system that local contraction causes a direct local deformation of local functional significance; as in a peristaltic ring of contraction. But in addition to its direct local effect, a contraction will cause an indirect deformation of other parts of the body through displacement of 'skeletal' fluid. In some reactions of animals with this class of skeletal system, the functional objective is reached primarily through the indirect consequence of contraction. We have noted earlier that a small contraction of the circular muscle of *Metridium* will, for mechanical reasons, cause a much larger elongation of the column. A more striking example of this effect is seen in the extrusion of the proboscis in the Nemertine worms where a small contraction of the circular musculature leads to the extrusion of a proboscis several times the length of the animal (e.g. *Geonemertes dendyi*, Pantin, to be published). Another elegant example is the action of the compensation sac in the eversion of the polypide in the cheilostomatous Polyzoa (Harmer, 1929).

The fact that contraction of a muscle in such systems has two consequences, direct and indirect, is of far-reaching importance. It is true that in a Vertebrate any movement of the limbs involves the co-ordinated action of many muscles. But in *Metridium* the movement of any muscle affects the mechanical conditions under which operates every other muscle of the body. A local contraction causes a rise in coelenteric pressure, and every other muscle must now do more work if it is to shorten than was necessary before. As we have seen, a high state of activity may actually reduce movement by raising the coelenteric pressure towards the isometric level. It is as though in a Vertebrate the contraction of one limb muscle necessitated a rise in tone in every other muscle in the body. The jointed structure of a Vertebrate or Arthropod, by localizing reciprocal muscular action, enormously increases the power of independent action of the parts of the animal. It has been suggested to us by Dr R. H. J. Brown that this same fundamental advantage may be found even in animals like the Annelid worms which are devoid of a jointed skeleton; for in this case segmentation breaks up the hydrostatic skeleton into a series of units with consequent localization of reciprocal muscular action. This may well be the most important of the several advantages conferred by the segmental plan. The only group of animals which has achieved substantial localization of reciprocal muscular action without the aid of a jointed skeleton or of segmentation is the Cephalopoda: it is one of their many morphological triumphs.

But it is clear that in the unlocalized hydrostatic skeletal system of *Metridium* there

is every advantage in the basal coelenteric pressure being as low as possible. The required movement of the parts can then be performed with the least expenditure of energy. A natural lower limit is set by that pressure which is just sufficient to prevent the collapse of the tissues under their own weight. We have seen that the basal pressure of *Metridium* is just above this, though collapse may sometimes occur during extreme expansion. For the maintenance of such a low basal pressure the 'trickle charge' by which the siphonoglyph fills the coelenteron seems admirably fitted.

Animals which live under greater conditions of mechanical stress, either because they burrow like *Arenicola*, or because they are more active or of larger mass, must make some sacrifice of mechanical efficiency to maintain their necessary basal turgor. Beyond a certain point this sacrifice must be too great for any advantage gained, and in larger and more active animals we repeatedly find the evolution of new or modified skeletal systems the mechanical analysis of which would very well repay further study.

SUMMARY

1. In contrast with most other Actinians, *Metridium senile* exhibits a great variety of shapes of the body. These are brought about by continual slow muscular activity.

The mechanics of muscular action are discussed. The action of most of the muscles is extremely slow. An isotonic contraction of the parietal muscles requires 40–60 sec. to reach its maximum and many minutes to relax. The body wall is capable of extension by about 400%. There are limits to extensibility in the normal animal. The mechanisms by which the animal itself increases or reduces extension by controlling its coelenteric volume are described. Fluid is gained chiefly through the siphonoglyph, though under certain conditions there may be suction into the coelenteron. Fluid is lost chiefly through reflex opening of the mouth. From time to time *Metridium* empties itself of fluid, and then refills in a few hours. A rate of refilling of 14 c.c./hr. has been measured.

2. Pressure changes in the coelenteron which occur during activity show that both retraction and extension of the column are active processes involving a rise in pressure which enforces reciprocal extension of the opposing musculature.

3. The relation of normal activity and shape to the coelenteric pressure is shown. This average pressure is extremely low; about 2–3 mm. of water. In a moderately filled unstimulated animal the natural muscular contractions are accompanied by a rise in pressure not generally exceeding 6–7 mm. of water. In such animals the natural contractions are of considerable extent, reaching over 30% of the body length.

4. By experimental inflation of the coelenteron with sea water, the system can be made to work more isometrically. The extent of movement is reduced and the animal may appear inactive. The presence of considerable though ineffective muscular activity is shown by the fact that large pressure changes (up to about 12 mm. of water) now take place. By raising the coelenteric pressure increased contractile activity in the body wall may actually reduce the extent of movement.

5. The isometric pressure which the body wall can develop in the coelenteron has been estimated. Pressures developed during natural contractions of a moderately filled animal demand muscular tensions in the body wall ranging between 20 and 50% of the isometric tension. The range of tension corresponds to that which would be most mechanically efficient if *Metridium* muscle resembles that of other animals.

6. An estimate is deduced from the coelenteric pressure of the isometric tension developed by the circular muscle of the column of *Metridium*. It is about 3·5 g./cm. of body wall transverse to the muscle. This is in agreement with direct observation of the isometric tension developed by strips of circular muscle. This tension in the column may correspond to a tension of 40 kg./sq.cm. of the individual muscle fibres and is very much greater than the values obtained from the frog's sartorius.

7. The extensive responses of the powerful retractor muscles involve much greater pressures (40–100 mm.) than those against which the column muscles can operate. The development of these muscles is related to the necessity of speed of action in a system undergoing great deformation.

8. Muscular action in a hydrostatic skeletal system is contrasted with that in the jointed skeletal system of Vertebrates and Arthropods. The former system is characterized by slowness of action and great change of length. In contrast with the Vertebrate skeletal system, in the hydrostatic system reciprocal muscular action is not localized. The movement of every muscle influences the mechanical conditions of every other in the system. Each muscle has two actions, a local direct action, and an indirect action, as in the elongation of *Metridium* on contraction of the circular muscles. The consequences of this are discussed.

Much of this work was done at the Marine Biological Laboratory, Plymouth, to the Director and staff of which we are most grateful for the many facilities given us. Part of the work was done during the tenure by one of us (E.J.B.) of a Shirtcliffe Fellowship of the University of New Zealand. We wish to thank the Department of Scientific and Industrial Research for a grant for the development of a special research which enabled this work to be concluded.

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BATHAM AND PANTIN—MUSCULAR AND HYDROSTATIC ACTION IN THE SEA-ANEMONE *METRIDIUM SENILE* (L.)

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EXPLANATION OF PLATE 7

States of *Metridium senile*. Photographs all to same scale. *a–f*, same individual on different occasions; *a*, enlarged volume after feeding; *b*, radial contraction of primary mesenteries; *c*, anemone in locomotion towards left; *d–f*, states of contraction and expansion; *g*, peristaltic wave in another, similar, specimen; *h–j*, 'shrivelling' followed by re-inflation: for description see text.

INHERENT ACTIVITY IN THE SEA-ANEMONE, *METRIDIUM SENILE* (L.)

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(With Six Text-figures)

A study of the anemone *Calliactis parasitica* (Pantin, 1935) showed that its most obvious activities are of a peculiarly simple kind. The unstimulated animal seems to be inactive. Activity originates directly as a response to external stimuli. Thus an adverse mechanical stimulus to the passive animal sets up impulses, transmitted by a special 'through-conduction system' which in this species activates the marginal sphincter muscle of the oral disk. The closure of this muscle protects the disk. Again, when food touches the tentacles, it is conveyed to the mouth by a series of local responses. In both these instances the activity consists of responses directly related to external stimuli, and the animal behaves as a passive vehicle for the conduction of excitation.

Similar direct responses to stimuli are to be found in the feeding reactions of *Anemonia sulcata* (Pantin & Pantin, 1943), and in both the feeding and the protective reactions of *Metridium senile* (Hall & Pantin, 1937). Parker (1919) noted the apparent absence of spontaneous and of rhythmic activity in *M. marginatum*. Direct observation would at first suggest this to be true of *M. senile*. But careful and prolonged observation by various methods reveals that the animal is in a state of continual and varied activity, which is, however, too slow to be easily perceived directly by the eye (Batham & Pantin, 1950). The investigations which we will now describe show that a large part of the behaviour of these animals is based upon activity which is not a direct response to external stimuli. It is inherent in the apparently unstimulated animal.

The character of the activity varies from time to time, the animal passing through different 'phases' of activity, which may endure for a considerable period. Thus the activity of an animal in a phase of elongation may differ greatly from one in a phase of general contraction. Hence there are two sets of phenomena to be investigated: the continual activity itself, and the variation of this activity with the phase of the animal.

The observed changes in shape which constitute the activity of the animal are brought about by muscular action. In a system such as we have here, the relation of muscular action to change of form is not as simple as it is in the body of an animal with a hard skeleton. The mechanics of muscular action in *Metridium* are discussed by us in a previous paper (Batham & Pantin, 1950). We will merely point out here that the main body of the anemone, the column, consists of a plastically extensible

cylinder whose walls carry a sheet of circular muscle and a system of longitudinal parietal muscles (see Fig. 1 of the paper quoted). The ends of the cylinder are closed by the oral disk and the attached foot. Between these run longitudinally the numerous mesenteries, the majority of which bear longitudinal retractor muscles. These various muscular sheets act upon the fluid contained in the gastral cavity; contraction of one layer thus tending to enforce distension of the others. The pressure developed in the gastral cavity is extremely low (1–6 mm. of water), and normally muscular action produces the maximum spatial displacement with the minimal performance of external work.

METHODS OF RECORDING

Three complementary methods of observation have been used: (i) prolonged direct observation; (ii) cinematography, particularly at slow speeds, about one exposure every $3\frac{1}{2}$–4 sec. ('time-lapse' cinematography); (iii) smoked drum records. Each method has special advantages. Direct observation leaves the animal less disturbed than with any other method. It is, however, difficult to obtain quantitative data in this way and continual observation over periods of hours or even days is exacting to the judgement of the observer. We have found that the value of such observation is greatly increased by viewing the anemone against a grid of squares behind it, and by making freehand sketches of the animal every half minute or minute over long periods. Cinematography not only gives permanent records, but when a film is viewed the apparent activity can be accelerated so that imperceptibly slow movements can be easily interpreted. When the apparent speed of events is increased in this way about 60-fold, *Metridium* seems to be a very active animal; and it is remarkable that the speeded movements of the animal as seen on the screen have a decidedly purposive character.

Smoked drum records are invaluable for obtaining quantitative measurement of the time relations and extent of movement. For the production of such records it is necessary to remember that the body executes contractions of great size, but that the tensions exerted by the muscles are normally extremely low. As we have said, the pressure in the coelenteron is only to be measured in millimetres of water. Consequently recording levers must be both free to move and so lightly balanced that they cause minimal deformity of the weak body. Happily, the movements are also slow, and they are therefore easily recorded by long isotonic levers writing lightly on rather thinly smoked paper. In all the figures the records read from left to right, and unless otherwise stated contractions are registered downwards.

With an animal capable of such great change of shape as *Metridium*, drum records must be continuously accompanied by direct observation. Only thus can a recorded movement be surely attributed to a particular system of muscles and not to the indirect consequence of some other deformation.

The attachment of a recording lever to an animal even of the simplicity of an actinian at once raises the question of how far the activity is 'normal' and how far this treatment is itself a source of stimulation. This kind of difficulty is also present in cinematography of the animals, and even to some extent with direct observation.

For in both these cases light is needed, and the animals show some degree of sensitivity to light. The difficulty is naturally acute when it is necessary to determine the behaviour during darkness. Fortunately, comparison of observations by the different methods, and particularly comparison with extensive direct observations both in aquaria and in the field made over the course of three years, leads to the conclusion that provided care is taken to have fine recording levers on the one hand, or to use constant lights of comparatively low intensity on the other, there is no obvious influence of the method of recording on the essential character of the behaviour.

Throughout this paper it must not be forgotten that we are dealing with a whole animal. We are not employing an isolated 'physiological preparation', the activity of which can be easily compared with that of controls 'at rest'. As in other 'whole' animals, the activity may vary even under apparently constant conditions, and a response to a stimulus may include a large and variable component supplied by the animal of itself. Often, therefore, a causal relationship between activity and environmental conditions is difficult to establish or to disprove except after prolonged observation of many instances such as we have made.

Most of the experiments were conducted at the Marine Biological Laboratory, Plymouth, on *Metridium* collected locally, particularly from Millbay Dock. During experiments they were kept in large vessels (2–10 l.) of clean, aerated sea water. Experiments were performed in an ordinary laboratory at room temperature and also in a rock-hewn cellar virtually free of vibration and under constant conditions of light and very small temperature fluctuation.

ACTIVITY OF THE COLUMN

We shall show elsewhere (Batham & Pantin, 1951) that the body of *Metridium* may be divided into three more or less separate systems of muscles. Of these, the muscular system of the foot is rarely active; that of the oral disk commonly shows some activity; while the muscular system of the column is always active. In the present paper we shall particularly consider this last system.

Fig. 1 shows the simultaneous activity of various parts of an unstimulated anemone, recorded isotonically. The lowest tracing (*C*) records the activity of the mesenteries by means of a thread attached to the lip of the mouth. The two tracings *A* and *B* above this record the vertical activity of the parietal musculature by threads from two opposite sides of the body wall. In *A*, *B* and *C* a contraction is registered downwards. Above this is recorded the activity at the marginal sphincter by a lateral thread (*Y*), the movement of which was transmitted to the recording point by a right-angled lever.

The uppermost tracing (*X*) records contraction of the circular muscle at a point about half way up the column. In this last case the tracing was recorded from the movement of a pivoted horizontal arm of straw resting lightly against the column of the anemone. This method was employed here because it was found that threads inserted into the column occasionally cause a curious inhibition of the circular muscle at that level so that a ring-like 'shelf' of weaker tone bulges out all round the column. This 'shelf' seems to act as a barrier to the conduction of peristaltic waves

up or down the column. The records begin with a retractor contraction following a brief stimulus, indicated on the time scale.

It will be seen at once from Fig. 1 that all parts of the column system are in continual activity. It will also be seen from the time scale that the activity is extremely slow. This applies to all the muscle systems including those of the mesenteries; though the latter can also respond with much greater rapidity to strong stimulation which initiates the retraction response. It will be shown in a later paper that there is some evidence that the mesenteric retractors, and perhaps also the sphincter, can contract at two different rates; though whether this is due to the possession of two distinct kinds of muscle fibre in the muscles is unknown.

The pattern of activity varies in different animals, but that recorded in Fig. 1 is quite commonly to be seen whether or not the animal has levers attached to it. The



Fig. 1. Kymograph tracing of activity of unstimulated anemone, recorded isotonically from the five points indicated in diagram at right. Arrows show direction of contraction. *X* is recorded from pivoted straw touching side of animal, as indicated in diagram.

major events are more or less periodic contractions of the parietal body wall. Each of these is then followed by an elongation of the column. This is not a mere passive relaxation of the parietals but an active extension due to contraction of the circular muscle layer (Batham & Pantin, 1950). The parietal contraction, which takes about 1 min. to reach its maximum, is followed by a slow contraction of the circular muscle immediately below the marginal sphincter. From the sphincter, a wave of contraction of the circular muscle now spreads down peristaltically, narrowing the column and causing it again to elongate. The successive elements of this sequence of contractions vary greatly in their intensity and their duration in different cases; and the activity may be complicated by the occurrence of more than one parietal or circular contraction in succession. Indeed, there is generally a background of irregular, though less intense, activity.

A characteristic pattern of events is shown in Fig. 2, recorded on a faster drum than that used for the record shown in Fig. 1. Contractions are registered downwards. The record shows the activity of a specimen displaying slight parietal

contractions and vigorous activity of its circular musculature. It will be seen that each parietal contraction (*a*, *b*, *c*, *d* and *e*) is followed about 0·5–1 min. later by a constriction of the sphincter region. This initiates a peristaltic wave which, in passing down the column, records itself at *X* between 1 and 2 min. later. However, such a sequence is by no means invariable. For instance, at *O* a spontaneous contraction of the circular muscle arose locally, without any preceding contraction of the parietal or sphincter.

These features of naturally occurring activity in the unstimulated animal are also to be seen when a response is elicited by electrical stimulation. We have shown (Batham & Pantin, 1950) that a battery of stimuli at low frequency (1 in 3 to 1 in 10 sec.) causes a contraction of the parietal musculature, which is followed by one or more waves of peristaltic circular muscle contractions over a period of several minutes.

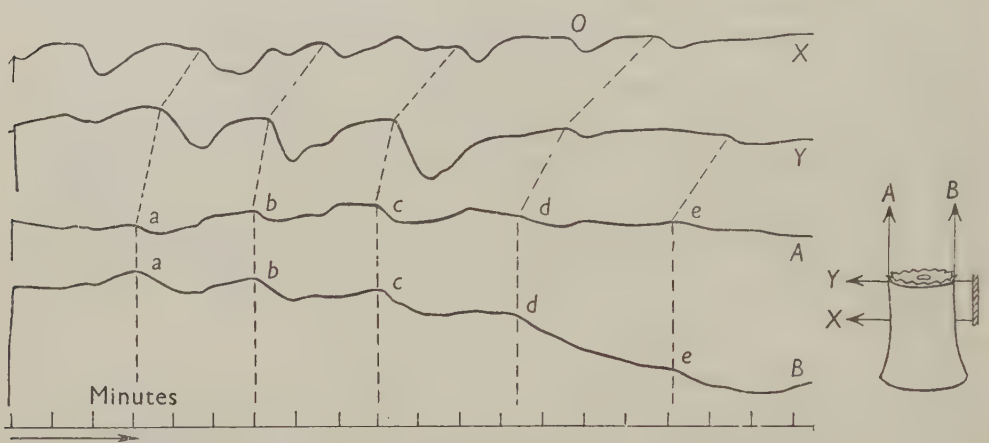


Fig. 2. Kymograph tracing of activity of unstimulated anemone, recorded isotonically on a faster drum than that used in Fig. 1. Contraction represented by fall in each tracing, whether recorded vertically (*A*, *B*) or horizontally (*X*, *Y*). Anemone attached to support on right by two fixed threads. *a–e*, parietal contractions. Broken lines indicate sequence of contractile activity. *O*, independent circular contraction.

RHYTHM AND CO-ORDINATION

One of the most striking features of the column activity is its frequent semi-rhythmic character. Fig. 1 shows the fairly regular occurrence of major parietal contractions at intervals round about 10 min. Approximate rhythms of about this frequency are often to be seen. Their low frequency is remarkable.

But the rhythm is sometimes entirely absent. Fig. 3 shows essentially a-rhythmic activity of three parts of the parietal musculature recorded from points round the upper edge of the body wall. Though a-rhythmic, the activity illustrated in Fig. 3 shares one important character in common with the rhythmic activity seen in Fig. 1: the activity of each part shows evident co-ordination with the rest. Such co-ordination is by no means complete. The parietal contractions recorded from the two points of the body wall in Fig. 1 do not all correspond. Rather more are to be seen

originating on the side recorded by tracing *A* than on that recorded by tracing *B*. Lack of co-ordination is almost always evident in the smaller contractions, particularly of the circular muscle layer. At times, large unco-ordinated contractions very obviously occur. The asymmetric activity of the parietal musculature recorded in Fig. 4 caused the animal to bend right over to one side.

Co-ordination is greater between different parts of the parietal system than it is between the parietals and the mesenteric retractors. The middle region of the column is apt to show the greatest departure from co-ordination (Fig. 5). The co-ordination between different parts of the parietal body wall may be surprisingly complete.

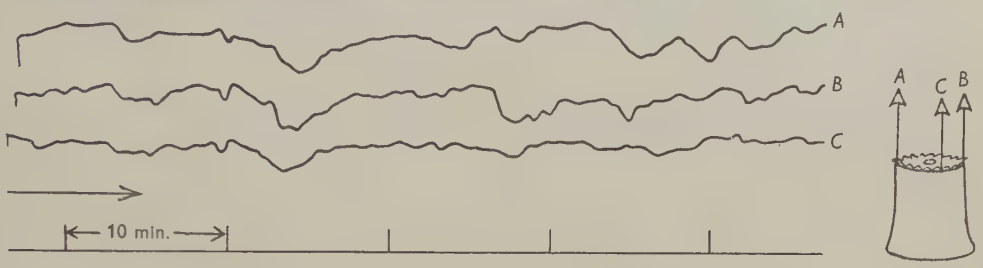


Fig. 3. Kymograph tracing of activity of parietal musculature recorded from three points of unstimulated anemone. Example of partially co-ordinated activity without clear rhythm.



Fig. 4. Kymograph tracing of parietal activity of an unstimulated anemone recorded from two diametrically opposite points. Note large unco-ordinated parietal contractions causing bending.

In experiments of this kind it is important to guard by direct observation against the possibility that co-ordination is simply of mechanical origin due to the drag of one part of the body upon another. The most direct proof that this is not the case is found in the co-ordinated activity of strips of body wall partly severed from connexion with the rest of the animal. These experiments will be discussed in a later paper. We may note, however, in Fig. 5 that the co-ordination of different points of the parietal wall is greater than that between the parietal wall and the mid-point of

the column on the same radius; or between the wall and the mouth region which lies between them. Further, comparison of the activity of the two parietal regions of Fig. 1 shows that by no means all the contractions of one region are accompanied by a response of the other.

One feature of co-ordination is of especial interest: its extreme slowness. We have seen that contractile activity initiated in the parietals may take minutes before it reaches the circular muscle of the column as a peristaltic wave. But even the passage of the contractile wave round the parietal system is slow. The onset of contractions does not begin simultaneously at different parts of the body wall. In some parts they may not begin till more than half a minute after the 'leading' part has begun to contract. This may result in a temporary asymmetry of the body, and

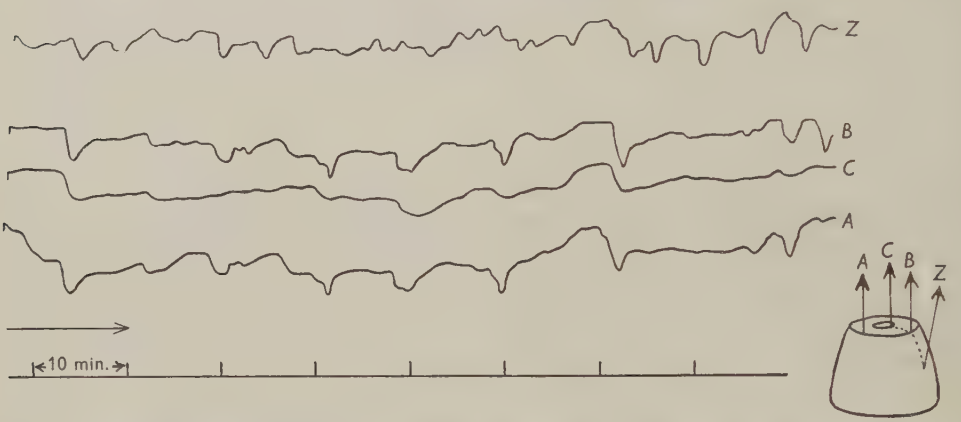


Fig. 5. Kymograph tracing of activity of unstimulated anemone, recorded from three points on the same radius and a fourth point (*A*) at 90°.

is another illustration of the independence between co-ordination and mechanical factors.

The following table shows the succession of the natural contractions of two parts of the body wall (Table 1, *A* and *C*), of the mesenteries at the mouth region (*B*), and of the sphincter (*D*). They were recorded in an anemone in a similar condition to that of Fig. 1, and with similarly arranged recording threads. The part which 'led' the contraction is recorded with the value 0, whilst the other figures show the number of seconds' delay before each part began to contract.

Table 1

A (Parietal)	*B* (Mesentery)	*C* (Parietal)	*D* (Sphincter)
4	0	10	30
0	20	15	38
0	17	22	30
5	15	0	50
7	0	17	45

These delays are far longer than can be accounted for by conduction of excitation in the nerve-net in the manner described by Pantin (1935) in *Calliactis*. Using the technique employed on that occasion, preliminary experiments were made by us on the conduction time of the through-conduction system of the nerve-net in *Metridium*. They were made by recording the latent period of contraction of the powerful retractors on opposite sides of the animal following stimulation by condenser shocks at a frequency of 1 per second applied to the base of the column. Values for the conduction time across animals some 5 cm. in diameter varied between 50 and 80 msec. Such values are larger than for the through-conduction system of *Calliactis*, but they are trivial in comparison with delays of the order of 15 sec. or more.

There is other evidence that these long delays are not simply due to slow progressive conduction across the nerve-net. If four or more threads are attached in order round the edge of the column, the order of the recorded responses of each sector of the column does not follow the order of their position. The part diametrically opposite the leader may often begin its response before the intermediate regions. It seems that a large part of these very long delays is of local origin and is not due to slow progressive conduction across the whole animal.

Table 1 shows that different parts of the anemone may act as 'leader' on different occasions. The observations illustrated here are rather exceptional in that 'leadership' more usually remains with one particular sector of the animal for long periods without change. It should be said in passing that direct observation shows that the 'leading' section of the column is as often located between two parts from which records are taken as not. There is no reason to associate initiation of activity with the trauma of an inserted recording thread.

INHERENT ACTIVITY

Sudden illumination or mechanical shocks may cause a response in an anemone. It is therefore necessary to consider whether the observed normal activity is really the simple consequence of random external stimulation. This does not seem to be so.

In the first place, animals placed side by side in the same aquarium show no correlation in the occurrence of their movements, except after evident common stimulation such as a large change in light intensity or the addition of food solution. In the second, removal so far as possible of external stimuli does not appear to affect activity. Animals were placed under comparatively constant environmental conditions in large bowls of clean sea water in a cool cellar. The bowls stood on shock-absorbing material on a firm base. The temperature of the water varied by about 0·5° C. over 24 hr., and readings during a day often differed by less than 0·1° C. Animals were left in darkness or in constant artificial light. The activity continued under these constant conditions, and indeed was not diminished in comparison with animals in aquaria on laboratory benches subject to variations of intensity of daylight or the ordinary mechanical vibrations of the room.

The activity was equally evident with direct observation or with cinematography of intact animals to which no threads were attached. The one form of stimulus which could not be avoided for very prolonged periods was the mechanical agitation

associated with aeration of sea water. Fig. 6, however, shows that absence of aeration produces no evident effect upon the character of the activity registered from the parietal body wall.

All these facts indicate that the activity is inherent in the animal and is not the result of successive responses to random external stimuli. This conclusion is fully borne out by the frequently rhythmic character of the activity (Fig. 1), for random stimuli will not recur at more or less regular intervals.

When we term the activity 'inherent' we do not imply that it is necessarily due to self-exciting elements, as in a self-exciting nerve or muscle cell or the rhythmically active ganglia which cause the regular excitation of the bell in Scyphomedusae (Bozler, 1926). The activity of *Metridium* causes changes of shape, and this raises the possibility that movement of the animal may itself excite subsequent movement after the manner of a chain reflex. It might be suggested that the rise in coelenteric pressure set up by a local contraction might automatically excite a stretch receptor or muscle fibre elsewhere in the animal. Parker (1919) has shown in *Condylactis* that sudden distension by fluid of an isolated tentacle may cause it to contract, and



Fig. 6. Isotonic record of parietal activity, whose nature does not change in the absence of aeration of the surrounding sea water by bubbles of air.

this is also true of the isolated tentacles of *Anemonia sulcata*. However, the pressures required to produce such contractions are considerable, whilst we have seen in the previous paper (Batham & Pantin, 1950) that active contractions are normally accompanied by extremely small changes of pressure (generally less than 6 mm. of water). Moreover, we found in the experiments recorded in the same paper that great distension of the coelenteron with fluid caused no general contraction. It sometimes caused a specific reflex contraction of the radial muscles of the mesenteries, but even this only occurred at very great distensions and, for the anemone, comparatively large pressures (40 mm. water). For a stretch to act as a stimulus in anemone tissue it must be very large—far larger than that which normally occurs during inherent activity.

It is difficult therefore to ascribe normal activity to a simple sequence of a response which engenders a stretch stimulus which in turn engenders a fresh response, after the manner of a chain reflex. Such an hypothesis would also have to contend with the difficulty of accounting for the fact that major contractions may occur regularly at long intervals of the order of 10 min. and that the intervening period between these contractions is spent in activity of a more irregular kind.

So far as our present observations go, they would be consistent with the supposition that activity arises spontaneously within the tissues. But while activity is evidently no simple chain reflex we cannot as yet certainly conclude that it arises entirely independently of stimuli or changes of excitability generated by the animal's own movements. It is for this reason that we have preferred to term the activity 'inherent'. We intend to imply by this simply that the activity is an observed property of the animals which does not arise directly from external stimuli.

From our experiments we may conclude that *Metridium* shows continual muscular activity in the column. This is so slow as scarcely to be perceptible to the eye as movement, and varies considerably in character and extent in different individuals and at different times. It is this activity which maintains the average state of tone balancing the coelenteric pressure (Batham & Pantin, 1950). The activity may involve a co-ordinated contraction sequence of the reciprocally acting circular and parietal muscles. Different parts of the parietal system may show co-ordinated contraction to a varying extent. The activity appears to be inherent and independent of external stimuli, and of the more obvious internal ones; such as stimulation by stretching of one part through contraction of another. The activity is often more or less rhythmic, giving fairly regular contractions at a frequency of the order of one in 10 min. Such very slow rhythms call to mind the rhythmic outbursts of organs composed of plain muscle such as the mammalian uterus and the proboscis of *Arenicola* (Wells, 1950). We have no evidence yet of the mechanism of the 'time keeper' in such slow activities.

Continual activity is to be found at times in all the muscular systems of *Metridium*, and not only in the column. Cinematograph records show that the tentacles of an open unstimulated quiescent anemone may exhibit either almost complete immobility or a continual active twitching. This contrast may be observed in two unstimulated anemones side by side in the same aquarium.

While *Metridium* by great modification of its shape shows activity very strikingly, even an apparently inert anemone like *Calliactis parasitica* is, in fact, in a state of continual activity. Prolonged records of the movement of the column of *Calliactis* show its state of continual though slight movement.

It will be shown later that inherent activity plays an essential part in the behaviour of these Actinozoa. It is no irrelevant by-product. In this we see an evident analogy with activity in higher animals possessed of a central nervous system. The activity cycles of Polychaete worms investigated by Wells (1950) provide an example.

The one striking contrast between this Actinian inherent activity and that of higher animals possessing a central nervous system is its extremely low frequency. Notwithstanding the similar part which inherent activity may play in both grades of nervous system, we must be careful not to suppose at present that identity of effect implies identity of mechanism; the systems differ too greatly in the degree of their structure, and in their time scale. The possibility that to achieve the same behavioural requirement, different kinds of process are utilized in the two grades of nervous system cannot as yet be dismissed (Pantin, 1950).

SUMMARY

1. The sea-anemone *Metridium senile* shows continual muscular activity. The activity is so slow that it is rarely appreciated by the eye as movement. Methods of observing and analysing such activity are discussed.

2. The activity of the column of the anemone has been analysed. It consists of a sequence of reciprocal contractions of the parietal muscles and the circular muscle coat. A sequence of activity commonly begins with a contraction of the parietals, followed by contraction of the marginal sphincter, which in turn initiates a peristaltic wave. The whole sequence lasts several minutes. The size and duration of its components may vary greatly. Activity may show a more or less regular rhythm with a period of the order of 10 min. between each major contraction. It may, however, show no trace of rhythm.

3. The activity of different parts of the body wall may show striking co-ordination. A contraction of one part of the parietal musculature is usually followed by contraction of the others. In other cases there may be no trace of co-ordination. The parietal muscles of one side may contract without contraction of those opposite, so that the animal bends over.

4. Co-ordination takes place through one part of the body wall acting as 'leader'. The other parts of the body wall follow this contraction with long delays (up to 30 sec. or more). The delay is far greater than the through-conduction time in the nerve-net (50–80 msec. in *Metridium*). There is evidence that it is of local origin. One sector usually maintains leadership for long periods; but from time to time the site of leadership changes.

5. Evidence is given that the activity continues unaltered in the absence of external stimulation. It is inherent. The evidence does not suggest that it is maintained through self-stimulation by preceding contractions after the manner of a chain reflex.

6. The activity varies greatly in character and extent in different animals and in the same animal at different times. This remains true even under apparently constant environmental conditions.

Much of this work was done at the Marine Biological Laboratory, Plymouth, to the Director and staff of which we are most grateful for the many facilities given us. Part of the work was done during the tenure by one of us (E.J.B.) of a Shirtcliffe Fellowship of the University of New Zealand. We wish to thank the Department of Scientific and Industrial Research for a grant for the development of a special research which enabled this work to be concluded.

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CHANGES IN THE BEHAVIOUR OF HONEY-BEES FOLLOWING THEIR RECOVERY FROM ANAESTHESIA

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INTRODUCTION

During the development of a marking technique which necessitated anaesthetizing honey-bees (*Apis mellifica* L.), certain after-effects of anaesthesia were noticed.

This work is a comparison of the after-effects of treatments with carbon dioxide, nitrogen and chloroform; it shows how behaviour patterns may be affected by physiological factors.

I am indebted to several of my colleagues at Rothamsted for criticisms and suggestions, and especially to Mr M. J. R. Healy for statistical advice.

METHODS

Bees were held in small specimen tubes with gauze-covered ends, exposed to chloroform vapour until they were anaesthetized, and then marked. Bees exposed to either carbon dioxide or nitrogen were dropped into tall glass jars which had been filled with the gas, kept there for 2 min. after anaesthesia, and then marked immediately. In warm weather they recovered quickly; when necessary, recovery was facilitated by placing them either in sunshine or in an incubator. Young bees were sprinkled with syrup when they recovered, and then dropped through the feed-hole in the crown-board of their hives; foraging bees were left in dishes on the lawn in front of the laboratory, and they flew away as they recovered.

Since the study was intended primarily to be qualitative no attempt was made to expose the bees to graded doses of anaesthetics. It is believed that variations in the activity of a bee at the moment of anaesthesia are likely to play an important part in determining the extent of the effects.

Bees were either group marked (each batch of bees all marked with a small blob of paint of one colour, so that they were recognizable as a group but not as individuals), or individually marked (each bee marked with a different symbol, and recognizable as an individual). For the latter method, with foraging bees, anaesthesia was necessary.

Modification of foraging behaviour by anaesthetics

On 14 and 16 May 1947, 90 bees were caught when they were returning to their hive with various pollen loads, and group marked; 40 untreated bees were marked blue, 20 CO_2-treated bees were marked white, and 30 $CHCl_3$-treated bees were marked yellow (Exp. 1). The hive was watched periodically on 17, 21 and 23 May, during which times the untreated controls returned from forty-nine trips, carrying

pollen loads on 34 occasions (69%), the $CHCl_3$-treated bees returned from 59 trips, carrying pollen loads on 36 occasions (61%), and the CO_2-treated bees returned from 24 trips, carrying a pollen load only once (4%).

On 29 May (Exp. 2), a further 196 bees, returning to their hive with full pollen loads, were caught and group marked. A N_2-treated group supplemented the treatments previously given. The hive entrance was watched for 2 hr. on each succeeding day until 11 June, on which date only one survivor was seen. The results are detailed in Table 1. These two experiments indicate that treatment with $CHCl_3$ does not

Table 1. Exp. 2. *Pollen-gathering capacity of untreated and treated bees*

Treatment	No. marked	Pollen presence or absence	Trips observed													Totals	Trips per bee	Percentage of trips with pollen
			May		June													
			30	31	1	2	3	4	5	6	7	8	9	10	11			
None	50	+	26	17	18	18	10	1	0	3	1	0	0	3	0	97	3·50	55
		−	21	9	14	8	7	4	5	5	0	2	2	2	0	78		
$CHCl_3$	45	+	23	16	20	11	11	2	1	6	0	1	2	0	0	93	3·56	58
		−	7	8	10	12	9	6	3	4	0	4	2	2	0	67		
CO_2	58	+	1	0	0	0	0	0	0	0	0	1	0	0	0	2	1·94	2
		−	34	13	24	15	7	0	2	6	3	4	2	1	0	111		
N_2	43	+	2	1	1	1	0	0	0	0	0	0	1	1	0	7	3·79	4
		−	37	19	23	18	14	6	6	13	5	7	3	5	1	157		

affect the pollen-gathering capabilities of the bees, but that pollen collecting almost ceased after treatment with either CO_2 or N_2. The flying activities of the CO_2-treated bees seemed consistently lower throughout the second experiment, but the treatment with either $CHCl_3$ or N_2 had no apparent effects upon either activity (as indicated by the number of trips per bee per day) or longevity.

These experiments conveyed no information concerning the extent to which the inhibition of pollen gathering might be associated with changes of crop or impairment of memory. It was therefore decided to treat bees which were working upon a row of cornflowers (*Centaurea cyanus* L.) from which they were collecting both nectar and pollen (Exp. 3). The bees were collected and divided at random into three groups of 15 bees each, which were treated with $CHCl_3$, and N_2 and CO_2 respectively, and individually marked, using a differently coloured set of symbols for each group. On the two subsequent days the cornflowers were inspected at half-hourly intervals, and the size of the pollen load of each individual forager was recorded. The presence of pollen was conclusive evidence that it was being collected, but, since any bee might have been seen at the commencement of her trip and before she had gathered a readily visible quantity of pollen, no conclusion could be drawn from its absence until this had been observed repeatedly. For this reason the records of two $CHCl_3$-treated and two N_2-treated bees, all seen on not more than two occasions, were omitted, and for each bee the maximum amount of pollen recorded was considered to typify its activities.

Of 11 $CHCl_3$-treated bees observed, 8 were seen with pollen loads, 2 carrying traces of pollen and 1 without pollen. (The number of observations was 97; 37 with pollen, 19 with traces, 41 without pollen). One of the two trace-collecting bees was

seen on 12 occasions (5 times with a trace and 7 without); this indicates the likelihood that there was a definite group of trace-collecting bees (possibly only collecting pollen accidentally acquired ?), and that some of these bees were not merely bees seen in the early stages of collecting a larger load.

The results from the CO_2-treated and N_2-treated bees were quite different. Of 6 CO_2-treated bees observed only 1 was seen with pollen, and 5 without (number of observations 48; 5 with pollen, 43 without pollen). Of 12 N_2-treated bees observed 1 was seen with pollen, 2 with traces of pollen, and 9 without (number of observations 77; 2 with pollen, 6 with traces, 69 without pollen). The differences between the results from the $CHCl_3$-treated bees and those from the CO_2-and N_2-treated bees were both statistically highly significant ($\chi_c^2 = 6{\cdot}4$ and $7{\cdot}6$ respectively; 1 degree of freedom (D.F.); probability (P) = 0·01).

Exp. 4 was a repetition of Exp. 3, using bees which were foraging upon patches of sainfoin (*Onobrychis sativa* L.). They were treated and individually marked on 27 July 1948, and the crop was inspected on the three subsequent days. Three groups of 12 bees were used.

Every one of the 8 $CHCl_3$-treated bees subsequently observed was seen to collect pollen (number of observations 129; 73 with pollen, 22 with traces, 34 without). None of the 5 CO_2-treated bees collected a pollen load, but one of them was seen with a trace load on the first day of observations (number of observations 62; 1 with a trace, 61 without). Of 8 N_2-treated bees observed, 2 collected pollen, 1 collected traces, 5 were without pollen (number of observations 67; 5 with pollen, 1 with traces, 61 without). The differences between the results from the $CHCl_3$-treated bees and those from the CO_2- and N_2-treated ones were both statistically significant ($\chi_c^2 = 5{\cdot}9$ and $4{\cdot}6$ respectively; 1 D.F.; $P = 0{\cdot}015$ and 0·03).

A feature of this experiment was that one of the $CHCl_3$-treated bees, which had collected pollen on two successive days (first day, 2 observations with traces and 4 without pollen; second day, 7 observations with pollen and 2 without) collected nectar only on the third day (5 observations without pollen). The absence of pollen collection on the third day was confirmed by watching this bee continuously for an hour and a half, during which time she obtained a nectar load from 1126 successive flower visits.

As these two experiments had shown that the inhibition of pollen gathering induced by CO_2 and N_2 was not necessarily associated with change of crop, it was decided to study the effects of the treatments upon bees foraging upon nectar-free pollen sources.

Shirley poppies (*Papaver rhoeas* L.) were therefore chosen for Exp. 5. On 31 July 1948, 55 Shirley poppy foragers were captured; 27 were treated with $CHCl_3$, 14 with CO_2 and 14 with N_2, and all were individually marked. The Shirley poppies were inspected at frequent intervals on the two subsequent days, and on 6 August a search for marked bees was made in the hives of an adjoining apiary, which had supplied a proportion of the foragers. The results, presented in Table 2, show that most of the $CHCl_3$-treated bees continued to return to the crop, but nearly all the CO_2-treated and N_2-treated bees deserted it, presumably in order to visit nectar crops elsewhere.

Table 2. Exp. 5. *Return of bees to Shirley Poppies*

Treatment	Bees marked	Bees observed on poppies		Bees seen alive in adjoining apiary
		1 Aug.	2 Aug.	6 Aug.
$CHCl_3$	27	17	14	6 (1 not seen on 1 or 2 Aug.)
CO_2	14	1	0	1 (not seen on 1 Aug.)
N_2	14	2	1	6 (5 not seen on 1 or 2 Aug.)

The effect of carbon dioxide and nitrogen anaesthesia upon the sequence of hive duties

The accepted pattern of honey-bee activity is based upon the researches of Rösch (1925 et seq.), who found that during their lives they undertook a sequence of duties, the last of which was foraging. Wiltze (1882), Nelson (1927) and Rösch (1930) have shown that the typical sequence can be modified to suit the requirements of the colony.

Rösch was unable to distinguish any definite sequence in the foraging activities, but in view of his observations there was a possibility that such a sequence existed, and that the changes in foraging habits after CO_2 and N_2 treatments might be a consequence of artificial ageing, and accompanied by changes in longevity. Exps. 6–9 were designed to test this hypothesis.

In each experiment either sealed brood or eggs of bees of one colour were introduced into a colony of bees of another colour. The approximate emergence dates of the introduced bees were calculated, and then confirmed by inspection. The young bees were then treated and individually marked, and their subsequent foraging activities determined by observations made at the hive entrance.

During these observations foraging trips, which were repeated at intervals, could be distinguished from occasional cleansing or exercise flights, which were single trips and on which the time between exit and entrance was usually less than two minutes. Foraging trips only will be considered in the presented results.

In view of Rösch's researches, the first foraging dates were divided into four periods:

(*a*) Bees not more than 12 days old = foraging within usual brood-rearing period.

(*b*) Bees 13–18 days old = foraging within usual wax-secreting period.

(*c*) Bees 19 and 20 days old = foraging during usual entrance guarding period.

(*d*) Bees at least 21 days old = foraging after completion of full sequence of hive duties.

The first of these experiments, no. 6, was a comparison of CO_2-treated and $CHCl_3$-treated bees. A comb of sealed brood of leather-coloured bees was introduced into a colony of black bees. 6 April 1948 is considered to be their date of emergence, but a few had emerged on the previous day, and some emerged subsequently. Between their fourth and seventh day 80 bees were treated with $CHCl_3$, and on the ninth day 30 bees were treated with CO_2; all these bees were individually marked.

Hive observations were made daily from the 10th to the 39th day, except on the

12th, 13th, 20th, 25th, 26th, and 27th days. The usual observation times were a 2 hr. period each morning and afternoon.

19 CO_2-treated and 55 $CHCl_3$-treated bees were observed. 5 out of 19 CO_2-treated bees but only 1 out of 55 $CHCl_3$-treated ones commenced foraging when less than 13 days old, during their normal brood-rearing period. All the CO_2-treated bees had commenced foraging within 19 days, but 51% of the $CHCl_3$-treated bees commenced on or after 21 days. Both of these differences are highly significant ($\chi_c^2 = 8{\cdot}3$ and $13{\cdot}5$; 1 D.F.; $P = 0{\cdot}005$ and $< 0{\cdot}001$).

The pollen-gathering propensities of the bees were also analysed. No significant differences were apparent between the proportion of pollen-gatherers among the early- and late-foraging groups of the $CHCl_3$-treated bees, but the two groups were separately recorded because the propensities of the CO_2- treated bees could only legitimately be compared with those of the $CHCl_3$-treated ones which commenced flighting at the same time, because forage available at different dates may have varied. Three out of 19 CO_2-treated bees and 15 out of 27 $CHCl_3$-treated early foragers gathered pollen loads on at least 10% of their observed trips. This difference is significant ($\chi_c^2 = 5{\cdot}8$; 1 D.F.; $P = 0{\cdot}016$).

It was decided to repeat this experiment with the addition of a group of N_2-treated bees (Exp. 7). A comb of eggs of black-coloured bees was introduced into a colony of golden bees. The date of emergence of the black bees was 1 June 1948 (1 day.). On 2, 3 and 5 June three groups, each of 20 bees, were individually marked after treatment with either N_2 or CO_2 or $CHCl_3$. Observations were impeded by inclement weather, and confined to 7–9, 14–17, 22, 23, 25 and 26 June and July 1–3, 6, 8, and 14 July. Twelve bees from each group were observed.

The dates on which foraging was first observed were:

(i) CO_2-treated bees: 9 bees < 12 days, 3 bees between 13 and 18 days.

(ii) $CHCl_3$-treated bees; 2 bees < 12 days, 4 bees between 13 and 18 days, 6 bees > 21 days.

(iii) N_2-treated bees: 9 bees < 12 days, 1 bee between 13 and 18 days, 2 bees > 21 days.

The differences between $CHCl_3$-treated bees and the CO_2- and N_2-treated bees are both significant ($\chi_c^2 = 6{\cdot}04$; 1 D.F.; $P = 0{\cdot}015$).

Pollen-gathering at this period was reduced and only nine loads were gathered by all the 36 bees with which we are now concerned, so no statement can be made concerning pollen-gathering propensities.

The relation between changes in behaviour and longevity

In Exp. 6 the expectation of life of the CO_2-treated bees was significantly reduced. Analysis indicated that this was not necessarily a direct effect of this treatment, but could be attributed to the change of behaviour which was induced. Comparison of the early foragers and late foragers among the $CHCl_3$-treated bees showed that although the mean foraging life of the former was greater than that of the latter ($15{\cdot}0 \pm 1{\cdot}2$ days compared with $10{\cdot}8 \pm 0{\cdot}8$ days) the mean total life was less ($30{\cdot}1 \pm 1{\cdot}2$ days compared with $37{\cdot}1 \pm 0{\cdot}6$ days). This implies that foraging life was

more hazardous and/or more exhausting than life within the hive. The mean foraging life of the CO_2-treated bees ($15{\cdot}1 \pm 1{\cdot}9$ days) was not significantly different from that of the early-foraging group of $CHCl_3$-treated bees.

The problem of longevity was investigated in greater detail in Exps. 8 and 9, in which eggs of black bees were introduced into a leather-coloured colony, and the black bees were collected from the hive entrance soon after they had commenced foraging. Half of these bees were treated with CO_2, and half with $CHCl_3$, and all were individually marked. Regular observations were made at the hive entrance, and the longevity was calculated from the date on which each bee was last seen.

In Exp. 8 the mean foraging life, from the date of marking, was $10{\cdot}2 \pm 0{\cdot}8$ days for 57 observed $CHCl_3$-treated bees and $10{\cdot}8 \pm 0{\cdot}8$ days for 64 observed CO_2-treated bees. The adequacy of the treatments is indicated by the fact that the former collected pollen on 21% of their trips, the latter only on 4%.

In Exp. 9 the periods were $10{\cdot}2 \pm 0{\cdot}4$ days for 50 observed $CHCl_3$-treated bees and $9{\cdot}8 \pm 0{\cdot}4$ days for 48 observed CO_2-treated bees. The former collected pollen on 12·3% of their trips, the latter only on 1·4%.

These results indicate that the CO_2 treatments, in the doses administered, had no direct effect on longevity.

Conclusions

Exps. 1 and 2 were considered to provide adequate evidence that the treatments with $CHCl_3$ had no after-effects upon either the pollen-gathering capacity or the longevity of the treated bees, and Exps. 3 and 4 showed that the memory of bees so treated was not impaired. $CHCl_3$-treated bees were therefore used in subsequent experiments as controls; such bees have since been used successfully in investigations of bee behaviour (e.g. Ribbands, 1949).

The conclusion that $CHCl_3$ anaesthesia did not impair memory, and that bees so treated returned normally to their own hives and to the crops which they had worked before treatment, conflicts with the view of von Buttel-Reepen (1900). He claimed that anaesthesia with either ether, chloroform or fumes of saltpetre destroyed all then-existing memories, because he was able to induce treated bees to enter hives not their own. Bees not completely recovered from anaesthesia will fan and crawl into any accumulation of bees; this fact would explain von Buttel-Reepen's results, and the conclusion which he drew from them was erroneous.

The experiments showed that CO_2 anaesthesia induced a permanent change in foraging behaviour, with the elimination or very marked reduction of the pollen-collecting tendency of bees so treated. Exps. 3 and 4 showed that this change was not associated with an impairment of memory for the crops which the bees had worked before treatment. The CO_2-treated bees returned to the crops which they had worked before treatment, but collected only nectar.

Another effect of CO_2 upon the honey-bee has already been recorded, by Mackensen (1947). He showed that queen honey-bees which have been instrumentally inseminated commence laying a week or more earlier after CO_2 anaesthesia than they do after some other anaesthetics, and that uninseminated virgins subjected to

similar treatment are soon caused to lay unfertilized eggs. Mackensen's results are complementary to those of Janisch (1924). Janisch exposed to CO_2 for short periods (less than 1 min) the females of three species of grain pests; he concluded that these insects were 'artificially aged', so that they went through their potential mating period, and that the treatments thus made them sterile.

Untreated worker honey-bees have a marked tendency to transfer their foraging from pollen sources to nectar sources (Ribbands, 1949), and it has frequently been supposed that the well-known sequence of duties within the hive is followed by a sequence of foraging duties. This result was associated with the results of Mackensen and Janisch, and led to the hypothesis that the CO_2 and N_2 treatments were artificially ageing the bees. Exps. 6 and 7, designed to test this hypothesis, proved that treatment of recently emerged bees with CO_2 induced them to forage at an earlier age than the $CHCl_3$-treated controls, so that all or a portion of their brood-rearing and wax-secreting activities were eliminated. Foraging life is more hazardous, and perhaps more exhausting, than life within the hive, and therefore the expectation of life of these newly emerged CO_2-treated bees was reduced. However, there was no observable difference between the longevity of these bees and that of those of the $CHCl_3$-treated bees used as controls which also went foraging at an early age.

Exps. 8 and 9 demonstrated that when the CO_2 treatments were given to bees of known age which had already commenced foraging they had no effect on their expectation of life. In addition, other experiments now in progress are indicating that there is no usual sequence of foraging duties in the field—some bees collect pollen throughout their foraging life, others never do, others sometimes do. For these reasons I conclude that the ageing hypothesis, initially so attractive, is incorrect.

Exps. 2–5 proved that N_2 anaesthesia also produced permanent partial or complete inhibition of the pollen-collecting tendency of foraging bees, and again the memory of the treated bees was not impaired. Exp. 7 suggested that N_2 treatments of newly emerged bees also induced them to forage at an earlier age than they otherwise would have done. There was no evidence of any direct effect of the N_2 treatments upon longevity.

DISCUSSION

The close similarity of the results of the CO_2 and N_2 treatments indicates that the effects are produced by the same mechanism in both cases. The factor common to both treatments is that they deprive the bee of oxygen; the effects may therefore be ascribed to oxygen lack. They are not produced by $CHCl_3$, which acts upon the nervous system. Dr Wigglesworth, in conversation, has suggested the possibility that oxygen lack prevents the oxidation of acid metabolities produced by the activity of the bee, and that temporary high accumulation of these metabolities gives rise in some way to permanent effects.

An important characteristic of the CO_2 and N_2 treatments is that they do not give rise to abnormal behaviour; they change the behaviour from one normal pattern to the other. Thus one control bee in Exp. 4 collected pollen and nectar on one day,

and nectar only on the next day, although her companions continued to collect supplies of both; again, when young bees were treated in Exps. 6 and 7 a few of the controls went foraging at an early age, just as all the CO_2-treated bees did. The results gain in importance from this conclusion, because in consequence the treatments may shed light on the normal mechanism through which the duties of the individual workers are regulated and integrated into a harmonious whole in accord with the requirements of each colony. They suggest a possible physiological basis of the observed behaviour patterns.

The outstanding attribute of the social insects, the differentiation and regulation of the activities of the individuals which compose the colony, has been the subject of much discussion. Roubaud (1916, 1924) and Wheeler (1918, 1928) have emphasized the predominant role of nutritional factors in the development of the community life of the social insects; their opinions were mainly derived from studies of wasps, ants and termites. In the honey-bee the few facets of the problem which have yielded to analysis have been shown to have a nutritional basis. Examples are the differentiation between queen and worker bees, and the subsequent 'nutritional castration' of the latter (Marchal, 1897), and the phenomenon of swarming which can be largely explained on the brood food theory which Gerstung propounded in 1891 (Morland, 1930). In another field, the time sense of bees is controlled by their rate of metabolism (Grabensberger, 1934; Kalmus, 1934). Now that the present experiments have shown that the regulation of some of the workers activities can be altered, apparently as a consequence of extreme oxygen lack, possibly future research will demonstrate the basis of the phenomenon of colony balance and the so-called 'hive-mind'.

The effects now recorded raise problems of practical importance as well as the theoretical ones so far discussed. There is a possibility that properly applied CO_2 treatments of whole colonies might prevent swarming, by converting the temporary excess of nurse bees into foragers, and also that they might be useful for increasing honey production in certain localities where brood rearing is said to be excessive, by reducing pollen collecting and brood rearing and increasing nectar collecting. Both the theoretical and the practical problems suggest interesting possibilities for future research.

SUMMARY

1. Chloroform anaesthesia did not impair the memory, change the foraging behaviour, or reduce the longevity of the treated bees; chloroform is therefore a satisfactory anaesthetic for use in experiments on bee behaviour.

2. Carbon dioxide anaesthesia did not impair the memory of treated bees, but it did induce a permanent change in their behaviour. Their pollen-collecting tendencies were either eliminated or suffered very marked reduction. Experiments with foraging bees of known age indicated that the carbon dioxide treatments had no direct effect on longevity. Treatment of recently emerged bees with carbon dioxide eliminated all or most of their brood-rearing and wax-secreting activities and caused them to forage at an early age. Foraging life is more hazardous than life within the

hive, and therefore the expectation of life of these carbon-dioxide treated bees was less than that of the controls.

3. The effects of nitrogen anaesthesia were similar to those obtained with carbon dioxide. The factor common to both treatments is oxygen lack.

4. The theoretical and practicable possibilities of these results are discussed.

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CENTRAL NERVOUS FUNCTION AND CHANGES IN BRAIN METABOLITE CONCENTRATION

I. GLYCOGEN AND LACTATE IN CONVULSING MICE

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(With Three Text-figures)

INTRODUCTION

For some time it has been known that the alteration of central nervous activity by various experimental procedures is accompanied by changes in the concentration of brain metabolites. The significance of earlier work in this field is discussed later in relation to the observations reported here. It may be noted, however, that the investigators have used a variety of techniques, and some of their results will have to be reconsidered in the light of improved methods of extraction.

The micro-chemical methods recently published by Kerr (1936) for glycogen and by Hawk, Oser & Summerson (1947) for lactate has made the estimation of these substances in small quantities both practical and reliable. The effect of a number of different procedures on the concentration of both brain metabolites in anaesthetized rats and rabbits has been made by Kerr & Ghantus (1936), and Stone (1938) has used one of the methods of lactate estimation for studying the effect of central nervous stimulants and depressants on lactate in the brain of mice. The object of this study is to enlarge the scope of these investigations by examining and attempting to define precisely the conditions in which any changes of either glycogen or lactate take place in mouse brain. Thus here we are concerned with whether the changes in these metabolites, which have been reported to occur by others during convulsions, are specifically related to this type of activity in the central nervous system, or whether changes in amount of glycogen and lactate are related to a number of different changes of nervous activity. These two substances have been chosen since it appears likely, on the basis of existing knowledge, that they undergo only one form of transmutation into other substances and can, therefore, be considered to lie in a biochemical cul-de-sac of carbohydrate breakdown. Thus they might be expected more easily to reflect by changes in their concentration any alteration of this metabolic process. While this work was in progress, Richter & Dawson (1948) have shown that an increase in lactate in rats appears to be associated with emotion. Thus evidence for changes of metabolite concentration specifically related to particular states of central nervous function already exists.

METHODS

Extraction

Samples in which the concentration of biochemical substances are to be estimated are usually now dropped alive into liquid air and are thus instantly frozen solid, after which the sample requires careful dissection. This method, however, is time-consuming, and in view of the large number of samples required by subsequent investigations, a method was devised for the rapid extraction of different parts of the living brain so that they could be transferred directly to the extraction fluid.

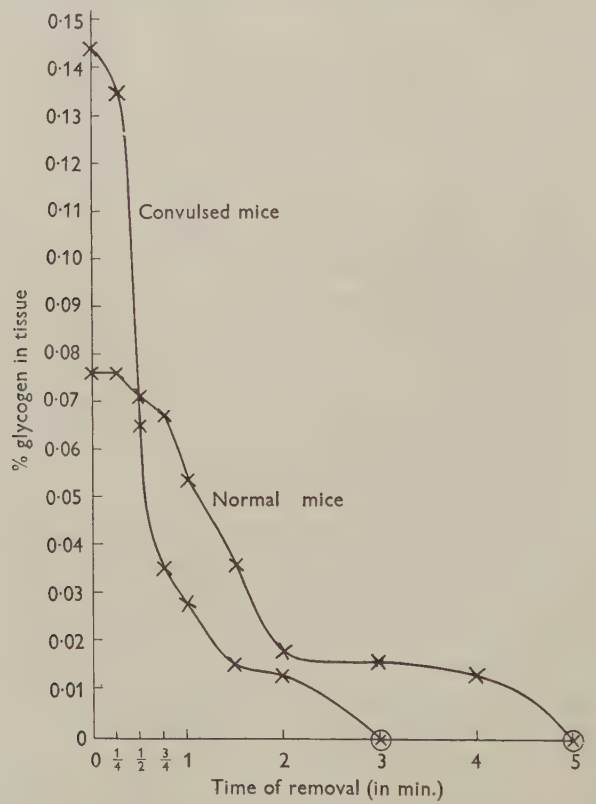


Fig. 1. Rate of loss of glycogen following circular-saw extraction of brain from normal and convulsed mice.

A circular saw driven by a $\frac{1}{16}$ h.p. motor running at approximately 2000 r.p.m. was used to cut through the head of the mouse, immediately after the animal had been decapitated. In this way samples from a variety of situations in the brain could be obtained between 10 and 20 sec. following the execution of the animal. To find out how long the concentration of glycogen and lactate remained at the level of the frozen samples after the head of the mouse had been dropped directly into liquid air, the values obtained by this method were compared with samples allowed to remain *in situ* for varying periods of time after decapitation. Fig. 1 shows the rate

of loss of glycogen and Fig. 2 of lactate in the mouse cortex following this procedure. From these it will be seen that samples removed during 30 sec. following execution retain the full amount of glycogen in animals which have not been convulsed, but

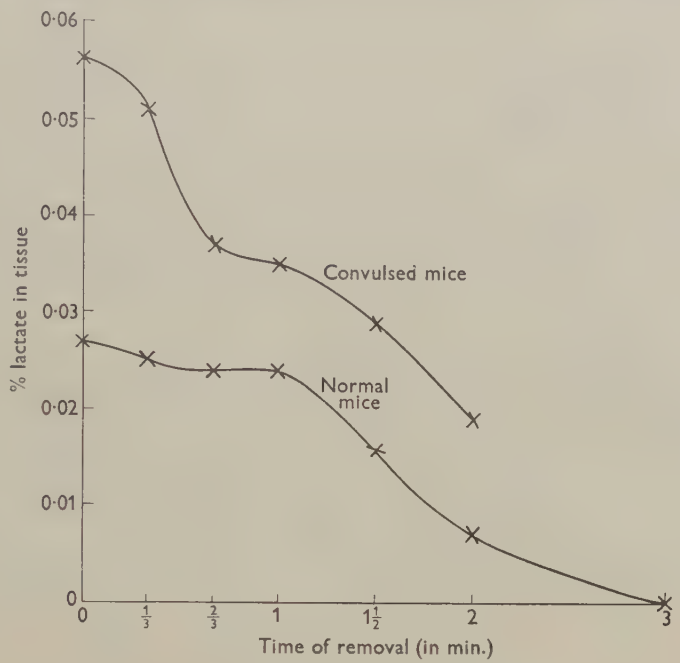


Fig. 2. Rate of loss of lactate following circular-saw extraction of brain from normal and convulsed mice.

Table 1. *Effect of method extraction on percentage increment of glycogen in convulsed mouse brain*

Means in heavy type.

Area	Drug	Dropped into liquid air				S.D.	Circular-saw extraction				S.D.	Diff.
Cortex	Picrotoxin	86	88	89	**87**	0·66	74	80	83	**79**	3·0	8
		86	88	87								
	Amphetamine	69	70	71	**70**		64	63	62	**63**		7
	Metrazol	86	85	85	**85**		82	80	76	**79**		6
Mid-brain	Picrotoxin	39	39	40	**39**		37	35	35	**36**		3
	Amphetamine	16	16	15	**16**		12	13	14	**13**		3
	Metrazol	53	52	53	**53**		49	49	50	**49**		4
Medulla	Picrotoxin	85	84	86	**85**		80	82	84	**82**		3
	Amphetamine	49	48	50	**49**		43	45	46	**45**		4
	Metrazol	64	63	62	**63**		58	59	61	**60**		3

that the glycogen concentration appears to fall approximately 15 sec. following death in those animals which have undergone an increase in the amount of glycogen present in the brain. It is thus satisfactory to estimate the glycogen directly in cortical tissue by means of the circular-saw method of extraction. Owing to the marked increase

in rate of loss of glycogen following procedures which increased the amounts of this metabolite in the brain, it was decided to check the validity of this assumption for the different parts of the brain following an increase of glycogen (Table 1).

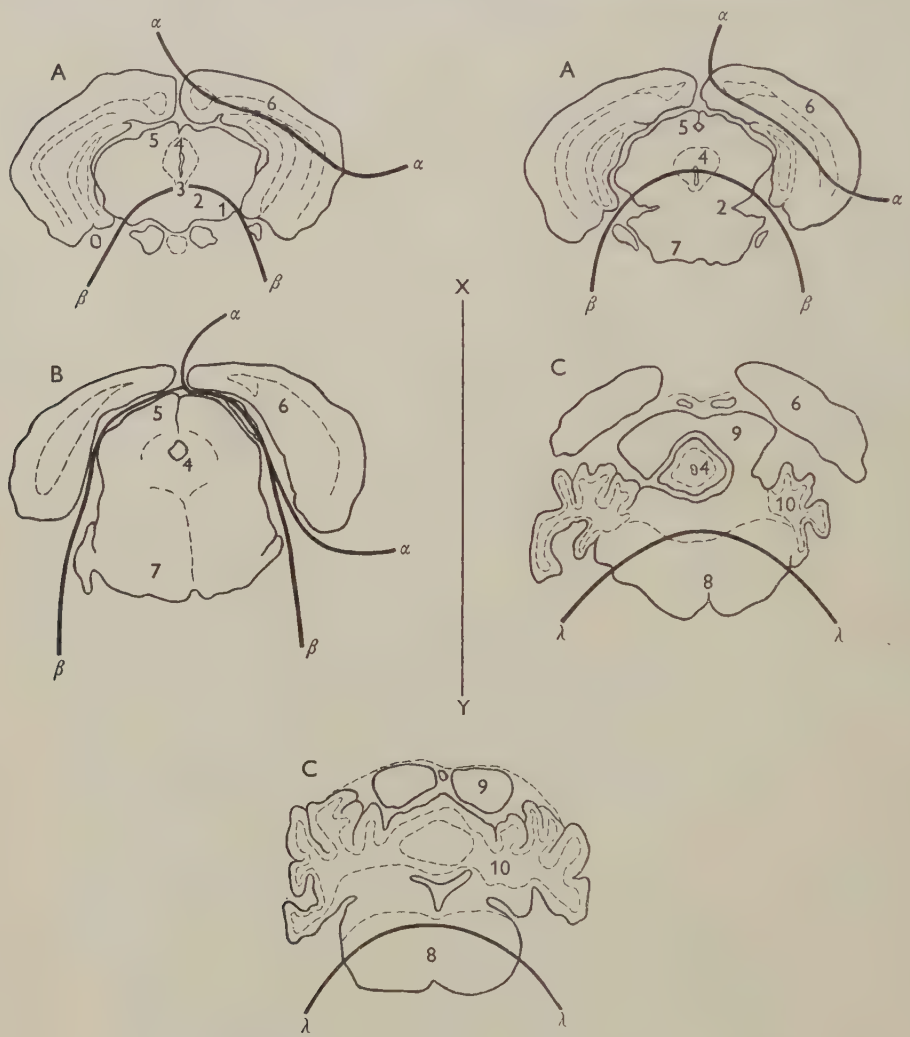


Fig. 3. Approximate anatomical limits of the three brain samples.

KEY. 1, substantia nigra; 2, red nucleus; 3, oculomotor nucleus; 4, central grey matter of the mid-brain; 5, superior colliculus; 6, cortex; 7, pons; 8, medulla; 9, inferior colliculus; 10, cerebellum. A, transverse sections mesencephalic region; B, transverse section metencephalic region; C, transverse sections myelencephalic region. α—α, cortical sample; β—β, mid-brain sample; λ—λ, medullary sample; X—Y, plane of transverse section across the head.

After the head has been removed it is cut transversely in the parieto-occipital region separating the brain between the met- and myelencephalon by means of the high-speed rotating saw. In this way the cortex and mid-brain can be separated as an anterior portion from the medulla and cerebellum (see Fig. 3). These parts can

then be sampled directly from the cut surface with a nickel spatula. The spinal cord can be sucked out by means of a glass capillary tube from the point where the head was severed from the body. The approximate limits of the regions sampled are shown in the figures of transverse sections of the mouse brain (Fig. 3).

These samples are transferred with their spatula or blown from the capillary tube directly into the extraction fluid in a previously weighed test-tube. On reweighing the weight of the sample is found by difference.

Estimation

Glycogen. The method of Kerr (1936), modified to increase the specificity of the method for estimating the reducing value, was used. The Schaffer-Hartmann method replaced the Somogyi method for the determination of reducing value, and the end-point was obtained with a sodium starch glycolate indicator according to the method of Bourne, Peat & Thrower (1947). Standard amounts of glucose were added from freshly prepared solutions of analar glucose when the sugar in exceptionally small quantities of brain tissue were estimated. By this means the reducing value could be brought on to the appropriate region of the curve for accurate estimation. The smallest quantity estimated by this method was 20 μg. The substance extracted and estimated was identified as glycogen by the following properties: acid hydrolysis, 76·5%; amylase hydrolysis, 39·1%; iodine brownish red; % yeast fermentable in convulsed brain, 97·2; % yeast fermentable in normal brain, 97·3.

Lactate. The method of Hawk *et al.* (1947) was used without alteration. The smallest quantity estimated by this method is 2 μg.

Experimental procedure

Convulsions can be induced in animals by either of two methods: (i) extrinsic methods involving the application of abnormal stimuli to normal animals, and (ii) intrinsic methods in which strains of animals with idiopathic susceptibility to seizures are subjected to normal precipitating stimuli. Both these methods have been studied. The extrinsic stimuli include electrical stimulation of the central nervous system by electrodes placed on the skull and on regions of the body close to the spinal cord, oxygen lack, and the action of drugs.

All these stimuli can be studied at subconvulsive and convulsive intensities.

Two rodent genera susceptible to seizures were available to us. *Peromyscus maniculatus*, obtained from Dr L. R. Dice, Ann Arbor, Michigan, U.S.A., produced convulsions on audiogenic stimulation. The pattern of their behaviour has been described by Chance & Yaxley (1949). We also obtained from Dr Hagedoorn a strain of *Mus* (H.C.) showing a dominant hereditary predisposition to convulsions of a different type in which the animal adopts a characteristic posture (Chance & Yaxley, 1951, unpublished) when lifted by its tail and gently shaken.

RESULTS

The normal values for the different regions of the brain obtained were in mg./100 g.:

	Glycogen	S.D.	No of. samples	Lactate	S.D.	No. of samples
Cortex	7·5	±0·38	22	26	±0·66	12
Mid-brain	27·5	±0·49	22	30	±0·60	9
Medulla	17·4	±0·56	22	35	±0·57	9

Increments of glycogen following convulsions induced by drugs (reported later) could be detected up to 40 min. after the end of the convulsion in animals killed after varying intervals of time. Approximately half the initial rise was present after 20 min. We have, therefore, made all estimations within a minute of the end of the convulsion.

Changes in lactate and glycogen under extrinsic stimuli

Stimulation by oxygen lack. The animal under test was placed in a small 250 c.c. bottle from which there was an inlet and an outlet through the rubber bung. In the first experiment nitrogen alone was passed over the animals, until after a few minutes apnoea developed and the animals died quietly. This procedure produced no alteration in the glycogen concentration, but a slight rise in lactate, as will be seen from Table 2. In the second experiment, the respiration was stimulated by the

Table 2. *Percentage increment of glycogen and lactate in mouse cortex during oxygen lack*

Means in heavy type.

	Pre-convulsive			Post-convulsive		
Procedure	Pre-convulsive symptoms	Glycogen	Lactate	Duration of convulsions (sec.)	Glycogen	Lactate
7 parts nitrogen, 1 part carbon dioxide	Escape behaviour	0 **0**	19 **23**	6	28 **27**	58 **71**
	followed by rapid	1	18	2 × 20	25	86
	shallow breathing	0	31	10	29	69
Nitrogen only	Dyspnoea to	1 **0**	15 **12**			
	respiratory	0	7			
	arrest	1	15			

presence of 12·5% carbon dioxide in nitrogen. The experiment was begun by mixing a small proportion of oxygen with this mixture, and the oxygen was then gradually reduced in amount until only the carbon dioxide and nitrogen remained in the inflowing stream of gas. By this method the animal survived very much longer, and after the respiration had become more rapid, in contrast with the result with nitrogen alone, clonic convulsions were precipitated which lasted from 5 to 12 sec. Following these convulsions the glycogen rose by approximately 30%, and there was a further increment of lactate.

Stimulation by drugs. In this experiment subconvulsive and convulsive levels of a number of different central nervous stimulants were used to investigate the relationship between convulsive seizures and changes in glycogen and lactate concentration. The dose used for the subconvulsive stimulation was two-thirds of the E.D. 50.* For the convulsive stimulation the E.D. 50 of the particular drug was chosen. As in the earlier experiment subconvulsive stimulation had little or no effect on the glycogen level except in the case of amphetamine, where a 4% increase was recorded; but this is not statistically significant. Here again changes in lactate accompanied both levels of stimulation (Table 3).

Table 3. *Percentage increment of glycogen and lactate in mouse cortex during stimulation by drugs*

Means in heavy type.

Drug	Sub-convulsive			Convulsive		
	Dose i.v. (mg./kg.)	Glycogen	Lactate	Dose i.v. (mg./kg.)	Glycogen	Lactate
Picrotoxin	1·6	−7 **0**	3 **6**	2·5	74 **79**	49 **44**
		4	17		80	26
		−4	−3		83	56
Metrazol	32	0 **3**	11 **16**	50	82 **79**	28 **28**
		6	17		80	34
		3	20		76	22
Amphetamine sulphate	40	4 **4**	15 **19**	60	64 **63**	30 **31**
		7	19		63	43
		1	24		62	20
		3				
		7				
		0				
		4				
		7				
		1				
Nikethamide	32	6 **2**	11 **14**	50	72 **74**	31 **36**
		1	7		76	34
		−1	24		76	44

Electrical stimulation. A 50-cycle current, the voltage of which was controlled by a variable transformer, was obtained by tapping the mains. The voltage and the current were measured simultaneously, and the electrodes were a copper bar in the mouth and a metal saline pad applied to the parietal and occipital regions of the head. In these experiments the voltage required for different animals was different according to the resistance of the animal and the contact which was made between the electrodes and the head. By variation either of the input or the degree of contact the current could be controlled. In Table 4, a number of different experiments were performed in order to obtain currents having three effects. When the lowest current was put into the animal by stimulation with 7·5 V., the animal struggled for 6 sec., then between 6 and 12 sec. tetany was induced with this voltage. Tetany was also maintained for 10 sec. by 5 V. in another experiment. In both these circumstances

* E.D. 50 equals C.D. 50 of other authors.

there was no change in glycogen concentration, although small changes in lactate occurred during struggling. These were greater during tetany.

Table 4. *Percentage increment of glycogen and lactate in mouse cortex during electrical stimulation through the head*

50 cycles a.c. electrodes through mouth and on parietal region of head.

Means in heavy type.

Nature of response	Sec. after stim.	Pre-convulsive mA.	Pre-convulsive Glycogen	Pre-convulsive mA.	Pre-convulsive Lactate	Convulsive mA.	Convulsive Glycogen	Convulsive mA.	Convulsive Lactate
20 V. producing 1 sec. tetany and 5–6 sec. convulsion		20	1 **0**	22	3 **2**	39	54 **55**	52	19 **26**
		26	1	28	1	46	55	56	26
		32	0	30		52	57	58	34
5 V. producing tetany for 10 sec.		25	0 **0**		19 **20**				
		26	0		23				
		30	1		17				
7·5 V.; mouse struggles for 6 sec. and passes into tetany for a further 5 sec., and after removal of the electrodes at 14 sec. has a 5–6 sec. convulsion	6	12	1 **0**	8	15 **12**				
		13	−1	10	3				
		15	0	11	18				
	12	10	5 **3**	10	29 **26**				
		15	3	11	29				
		19	1	16	21				
	20					14	86 **77**	12	56 **43**
						16	73	12	33
						17	73	13	40

A large increase in glycogen, however, occurred as soon as the current reached a sufficiently high value or was maintained long enough to obtain a clonic convulsion on removal of the electrodes. In these circumstances also the lactate rose to the highest value.

Tables 2, 3 and 4 show, therefore, that subconvulsive intensities of any extrinsic stimulus, whether by electrical stimulation, the action of drugs or oxygen lack, produce a slight but statistically significant increase of lactate, without a significant change in the amount of glycogen. When convulsions are precipitated by these means, however, there is usually a small further increment in lactate and the amount of glycogen rises sharply. A point of interest about these results is the constancy in the amount of glycogen which accumulates after any particular method of precipitating a convulsion, whereas the change in lactate is more variable.

Table 5 shows that for the same region of brain the accumulation in glycogen is greater for tonic than for clonic convulsions, when the electrodes are applied to the same region of the head. These increments of glycogen were accompanied by a slightly greater intensity of electrical stimulation of the brain required to induce a tonic rather than a clonic convulsion. Since the intensity of the electrical stimulation is related to the distance from the electrodes the effect of applying electrodes in such a way as to produce different intensities of stimulation in different regions of the

Table 5. *Percentage glycogen increment in mouse brain and severity of convulsions*

Electrodes on top of head and along spinal column.

Means in heavy type.

Procedure	Area	S.D.	Glycogen			
20 V. producing *tonic* convulsion after 3 sec. stimulation	Cortex	1·8	61	62	60	**61**
	Mid-brain		72	70	68	**70**
	Medulla		67	70	64	**67**
	Spinal cord		80	82	85	**82**
mA.			40	32	37	
15 V. producing *clonic* convulsion after 3 sec. stimulation	Cortex	1·5	57	54	58	**56**
	Mid-brain		64	62	63	**63**
	Medulla		58	63	61	**61**
	Spinal cord		80	79	77	**79**
mA.			32	31	33	

Table 6. *Electrical stimulation. Position of electrodes and percentage glycogen increment in different regions of the brain*

Means in heavy type.

I. Electrodes on top of head and roof of mouth.

Procedure	Area	S.D.	Glycogen			
15 V. producing *clonic* convulsion after 3 sec. stimulation	Cortex	1·1	67	64	66	**66**
	Mid-brain		71	74	70	**72**
	Medulla		71	70	68	**70**
	Spinal cord		60	61	62	**61**
mA.			28	32	30	

II. Electrodes on each side of head between eye and ear.

Procedure	Area	S.D.	Glycogen			
15 V. producing *clonic* convulsion after 3 sec. stimulation	Cortex	1·3	74	75	73	**74**
	Mid-brain		62	63	64	**63**
	Medulla		66	70	68	**68**
	Spinal cord		55	57	57	**56**
mA.			—	—	—	

Table 7. *Glycogen values in mouse brain resulting from prolonged nembutal anaesthesia*

Single doses daily (60 g./kg.) S.C. Anaesthesia lasting 2–3 hr. 20 g. male mice.

Duration of test	Medulla (% gly.)		Cerebrum (% gly.)		Mid-brain (% gly.)	
	Individual values	Mean	Individual values	Mean	Individual values	Mean
2½ hours	0·27 0·28 0·27	0·27	0·08 0·07 0·08	0·08	0·16 0·15 0·16	0·16
1 day	0·27 0·26 0·26	0·26	0·08 0·08 0·08	0·08	0·16 0·17 0·17	0·17
4 days	0·26 0·27 0·27	0·27	0·08 0·08 0·09	0·08	0·16 0·17 0·16	0·16
8 days	0·27 0·26 0·26	0·26	0·08 0·07 0·08	0·08	0·16 0·17 0·17	0·17
16 days	0·26 0·27 0·26	0·26	0·08 0·07 0·08	0·08	0·17 0·16 0·16	0·16
Under anaesthesia (approx. ¾ hr. after injection)	0·28 0·28 0·27	0·28	0·08 0·07 0·07	0·07	0·16 0·18 0·17	0·17

head would be expected to produce correspondingly greater increases in those regions close to the electrodes. This is shown in Table 6. The absence of any change in glycogen under nembutal anaesthesia or after prolonged periods of depression produced by this drug is shown in Table 7.

Changes in glycogen and lactate under intrinsic stimuli

As described in an earlier publication (Chance & Yaxley, 1949), the jingling of metal tubes induced escape reactions in animals of the strain of *Peromyscus* susceptible to seizures. First of all the animal rushed about in the container in a controlled manner, then it crouched and finally it passed into a phase of uncontrolled running which inevitably terminated in a clonic or tonic seizure. This happened in immature animals for a period of approximately 2 months from the time of weaning and ceased, first of all, by the suppression of the terminal convulsions, at about maturity. In view of the findings of the previous section, which showed that only when extrinsic stimuli were of sufficient intensity to produce convulsions was there any change in the glycogen level of the brain it was thought worth while investigating the effect of precipitating stimuli on the brain glycogen of non-susceptible and susceptible animals of the strain of *Peromyscus*. Accordingly, the glycogen level was estimated in the mid-brain, cortex and medulla of the young unstimulated *Peromyscus*, and in litter-mates subjected to audiogenic stimulation to the point of convulsions. All the animals of the litter had on the previous day shown the typical sequence of behaviour described above, ending in clonic convulsions followed by periods of automatism lasting between 15 and 40 sec. The same pattern was repeated by the animals on the day when the estimation of glycogen was made within a minute of the end of the convulsion and approximately 30 sec. after the end of automatism, in three litter-mates.

Table 8. *Glycogen values in* Peromyscus *brain under intrinsic stimulation*

	No. of animals	Young reacters			No. of animals	Old non-reacters		
		Cortex	Mid-brain	Medulla		Cortex	Mid-brain	Medulla
Unstimulated	2	0·110 0·126	0·185 0·200	0·320 0·305	2	0·082 0·078	0·185 0·180	0·275 0·285
Mean		0·118	0·192	0·312		0·080	0·183	0·280
Stimulated	3	0·216 0·210 0·221	0·240 0·280 0·230	0·560 0·520 0·540	2	0·094 0·097	0·167 0·140	0·280 0·291
Mean		0·216	0·250	0·540		0·095	0·153	0·285
% change of stimulated over unstimulated		+75	+30	+73		+18	−16	+2

These animals showed a marked increase in all regions of the brain compared with their litter-mates killed contemporaneously without stimulation (Table 8). It will be noted that stimulation of these animals after they had lost their susceptibility to seizures produced slight changes in the amount of glycogen in different parts of the

brain. This is the only instance we have found of alteration in glycogen level without accompanying signs of convulsions in the overt behaviour of the animals.

Momentary loss of support produced by suspending the H.C. mice by their tails and gently shaking them produced the typical convulsions described elsewhere. An increase in glycogen was found in the medulla and cortex and a slight increase in the mid-brain, but no increase occurred when the mice were shaken by the tail for a short period without precipitating a convulsion. Increases in lactate also occurred in these three regions during the convulsions (Table 9).

Table 9. *Glycogen and lactate values for H.C. mice brain under intrinsic stimulation*

	No. of animals	Glycogen			Lactate		
		Cortex	Mid-brain	Medulla	Cortex	Mid-brain	Medulla
Normal	2	0·078 0·076	0·190 0·180	0·200 0·220	0·027 0·023	0·030 0·026	0·037 0·039
Mean		0·077	0·185	0·210	0·025	0·028	0·038
Held by tail and shaken without convulsion	2	0·075 0·076	0·190 0·195	0·220 0·230			
Mean		0·076	0·192	0·225			
Held by tail and shaken till convulsed	2	0·128 0·134	0·206 0·195	0·345 0·310	0·031 0·033	0·032 0·035	0·042 0·045
Mean		0·131	0·200	0·328	0·032	0·034	0·044
% increment of convulsed over normal		+70	+8	+56	+27	+19	+15

DISCUSSION

In mouse brain glycogen has been estimated by Neiman (1936) and lactate by Stone (1938), and reports on both substances show that they have been estimated on frequent occasions in cats, dogs and many other species. Our results confirm the findings of Stone, who showed that convulsive and subconvulsive doses of central nervous stimulants produced increments of brain lactate. He also showed that depression of the central nervous system by anaesthetics and during sleep was accompanied by a lowering of the brain lactate. The same relationship between excitability and depression in the central nervous system has been found by Richter & Dawson (1948) working with rats, but the particular value of their work lies in the fact they have shown that only when this excitability is accompanied by an emotional component is there a significant increment in brain lactate. It would appear, therefore, that increments in brain lactate reflect an emotional component of a particular activity of the central nervous system, but that depression of the lactate level occurs in a number of different conditions engendering a lowered excitability of the central nervous system. If this is so, it is not surprising that we have found little correlation between lactate changes and any of the experimental conditions designed to produce convulsions. The contrary is the case with the glycogen in brain, and so we have

concentrated in the latter part of the investigations on estimations of this substance.

Except for the work of Kerr & Ghantus (1936) and Chesler & Himwich (1943), it is impossible to compare our results with the figures obtained by other workers. Until the publication of Kerr's paper in 1936 the methods used for extraction were unreliable because they involved long periods elapsing between the death of the animal and the extraction of the tissue (Holmes, 1926; Kobori, 1926; Takahashi, 1924, 1925; and Winterstein & Hirchberg, 1925). There is the added complication that none of them have worked with mice.

Uchida (1926) and Kojima (1927) give sufficient details of their methods of extraction to suggest that they obtained their material from rats and rabbits within a minute or two of the animal's death, though no precise information on this is given. The values obtained by them are of the same order as those obtained by Kerr & Ghantus for dogs and rabbits, but in view of the rapid loss we have found in mice, following removal from the animal, their results, though suggestive, are inconclusive.

Anaesthetization by itself would not appear to be a barrier in the way of obtaining normal glycogen levels since the values reported by Kerr and his colleagues who anaesthetized cats and dogs agree substantially with those we have obtained in mice and which were also found for rats and mice by Neiman (1936). From these comparisons we may infer that a large number of vertebrates normally possess between 10 and 30 mg. of glycogen per 100 g. of brain tissue, though as Kerr & Ghantus (1936) and Neiman (1936), as well as our own findings, demonstrate the exact amount in different parts of the brain of the same species show consistent differences; one part of the brain possessing as much as two or three times another.

On the other hand, it is quite possible that as anaesthesia seriously interferes with central nervous function it thus alters the effect of various procedures on the glycogen level of this tissue. For this reason it is not perhaps surprising that an increase of glycogen following convulsions has not previously been demonstrated. In particular, the work of Kerr & Ghantus (1936) and Klein & Olsen (1947) is unreliable in this respect because of their use of anaesthetics. The convulsions in each instance were induced in anaesthetized animals.

We have not been able to find conditions which will put up the amount of glycogen in the brain by more than 100%. Chesler & Himwich (1943) recommend the use of iodo acetate injections as a means of determining the glycogen concentration *in situ*. This method, however, gives a loss of some 25% in the brain glycogen in comparison with liquid air extraction and is therefore not sufficiently reliable for use in studying differences in the accumulation of glycogen in different parts of the central nervous system. On the other hand, the use of a circular saw, such as we have found satisfactory for estimations of samples of mouse brain, may prove impractible for larger animals. While the loss of glycogen obtained in this way is sufficiently small to allow comparisons of changes in different parts of the brain, it is our opinion that new methods should always be checked against extraction after freezing in liquid air, before the reliability of any procedure can be assessed.

Subconvulsive intensities of the intrinsic stimuli had no effect on the level of

glycogen in the H.C. strain of mice. In this respect they show the same characteristics as normal animals receiving extrinsic stimulation. The convulsion in the strain either appears or is absent, and the glycogen level follows the same pattern. The glycogen appears to be more labile in the audiogenic strain of *Peromyscus*; in these animals the convulsion is the terminal state of a progressive disturbance of behaviour. This may indicate the presence of convulsive autonomic discharges, which are not visible, or, alternatively, a residual susceptibility to convulsive discharge may exist in the older mice which is prevented from disturbing their behaviour by compensatory nervous pathways. The significance of the fall in glycogen in the mid-brain of these older animals is, however, still obscure.

SUMMARY

The content of glycogen and lactate in mouse brain has been estimated after a new extraction procedure which has been demonstrated to involve only a slight loss in amount of these two metabolites in comparison with extraction following freezing *in situ*. Five different methods of precipitating convulsions produced increases in the amount of lactate concentration at both subconvulsive and convulsive levels, but glycogen increased appreciably only when these methods produced a convulsion. It is concluded that glycogen increase in the mouse brain is specifically related to the occurrence of epileptic discharge in the brain. This increase has been shown to occur in all parts of the brain, though in varying degrees. Previous studies on these brain metabolites are discussed in the light of these results.

We are indebted to Prof. A. C. Frazer for his interest in the work, and one of us (M.R.A.C.) is indebted to Mr A. L. Bacharach for an early opportunity to explore the possibilities of this study. We should like to thank Dr E. J. Bourne for his advice on the method of extraction of glycogen, and Dr R. Gaddy for estimating the yeast fermentable reducing substance in the material extracted from the brain. We are also grateful to Mr A. E. Froggatt for the design of the high-speed rotating saws.

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RADIOLOGICAL OBSERVATIONS ON THE CARDIO-VASCULAR SYSTEM IN *ANGUILLA ANGUILLA*

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(With Plates 8–10 and Two Text-figures)

INTRODUCTION

Comparatively little work appears to have been done on the circulation in the intact living fish, particularly in the bony fish. This paper describes the circulation in the common eel using angiographic methods. This species is convenient as it can be fairly easily obtained in the autumn and winter, is tolerant of laboratory conditions and can survive for some time out of water. As no general description of the circulation in the eel appeared to exist, it proved necessary, in order to interpret the angiographs obtained in the living animal, to make a number of dissections, of which a general account has been given elsewhere (Mott, 1950).

MATERIAL AND METHODS

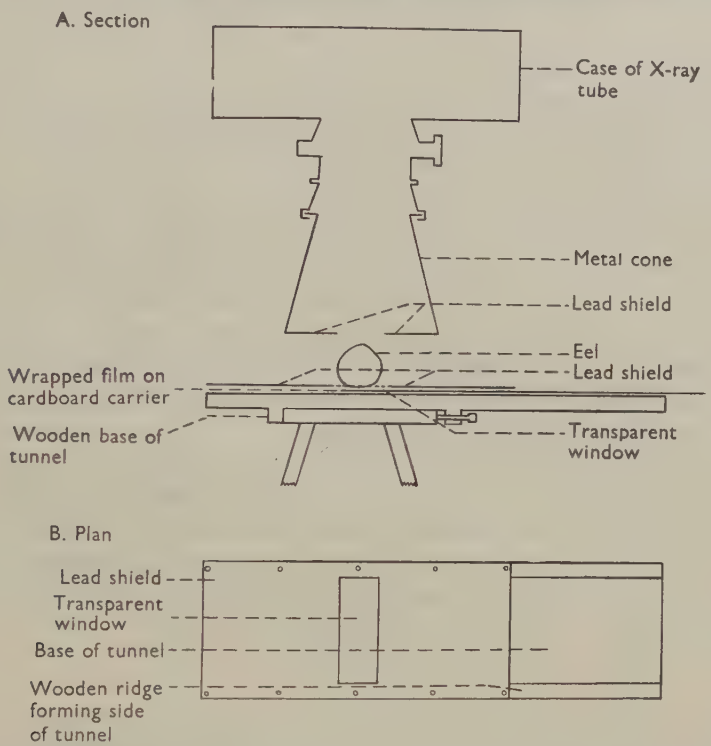
The silver eels used were obtained through the good offices of the Freshwater Biological Association, and were kept in a large tiled tank in running tap water.

The method involved the radiographic recording at frequent intervals of a radiopaque substance introduced into the blood stream. The techniques of indirect and direct cineradiography have been discussed by Barclay *et al.* (Barclay, Franklin & Prichard, 1944). For various reasons their techniques were not suitable for a small animal in which good definition of the vessels was of the first importance. The technique used consisted of the intermittent passage by hand of a strip of cardboard carrying wrapped Ilfex non-screen X-ray films through a shallow wooden tunnel, the top of which was made of sheet lead with a transparent plastic window on which the animal was laid, as in Text-fig. 1. The X-ray exposures were initiated by depression of a foot key which also operated a marker on a smoked drum revolving at a known speed, from which the intervals between exposures could be calculated. Exposures were made at any desired rate up to a maximum rate of about three in every 2 sec. in the hands of a practised operator. This system has now been superseded by a machine designed by Dr E. H. J. Schuster for moving the film at a rate of two exposures per second. Exposures were made at 35 kV. and 300 mA. Under these conditions, and at an anode distance of about 16 in., the exposure required was of the order of 0·1 sec.

The eel was anaesthetized by immersion in $2\frac{1}{2}$ % urethane until it was limp and had lost its righting reflex. In a few eels, when it was desired to increase the number

of angiographs obtainable during each heart beat, the animal was immersed in colder water to slow the heart. Otherwise, no attempt was made to control the temperature at which the experiments were performed, but the heart rate and temperature were noted at intervals during the experiment. The eel, in common with many fish, has no superficial veins convenient for cannulation. Two vessels were used for injection; the caudal vein for the circulation from the heart to the anterior gut arteries, and the ventral aorta, for the circulation of the gut and associated organs.

In order to cannulate the caudal vein the greater part of the eel's tail was removed and the caudal artery and vein clamped; this avoids a stagnant section of circulation



Text-fig. 1. Diagrams showing: (A) sectional view of apparatus for making serial radiographs; (B) plan of wooden tunnel through which wrapped X-ray film is pushed.

in the tail. The vein was approached through a mid-ventral incision and a metal cannula, filled with eel ringer (Keys, 1931) with a stopper in the proximal end, was tied in. When the injection was to be made, the stopper was replaced by a syringe containing Thorotrast (a colloidal suspension of thorium dioxide). Most of the eels weighed between 500 and 750 g., and 1 c.c. of Thorotrast proved adequate for reasonable contrast. The use of the caudal vein had two advantages:

(*a*) The injection was made well away from the heart and on the distal side of the hepatic portal and/or renal portal systems, so that the injection was unlikely to produce an abnormally high pressure in veins immediately adjacent to the heart.

In fact, no appreciable reflux was ever seen in any of the veins entering the sinus venosus.

(*b*) The operator's hands are well away from the X-rays.

Injections were made into the exposed ventral aorta using a fine curved hypodermic needle which was inserted into the vessel and tied to the neighbouring muscle. Thorotrast (1 c.c.) was again used for the injection and was introduced fairly slowly in an attempt to allow the normal flow in the ventral aorta to carry the injected material with it.

It was possible to make at least two injections in any one animal, but in the absence of any mechanism which eliminates Thorotrast from the circulation, such as is present in many mammals (Barclay *et al.* 1944), the Thorotrast previously introduced causes some haziness.

A few experiments were done under water in a plastic box with a thin bottom virtually transparent to X-rays, but there did not seem to be any difference from the results obtained in air. As the anaesthetic usually inhibits the respiratory movements of the opercula this is perhaps not surprising.

The eel was kept in water when anaesthetized, except when being radiographed and cutaneous respiration seemed to be quite adequate for maintaining strong and regular contractions of the heart for the duration of the experiments.

The interpretation of the angiographs required some practice, and considerable help was obtained from the anatomical studies already mentioned. The calibre of vessels was measured when required by a modified pair of vernier callipers devised by the late Dr Barclay (Barclay *et al.* 1944).

It proved difficult in some instances to distinguish bone and injected vessels, both of which cast shadows. One way of overcoming this is to make a 'positive' from the X-ray film of an earlier uninjected frame of the series and then print through both 'positive' and a later frame of the series. In this way, as in Pl. 8, fig. 1, a print is obtained showing vessels only; but the procedure is time-consuming and is not worthwhile except in special instances.

RESULTS

The circulation through the heart and gills as shown by the injection of Thorotrast into the caudal vein

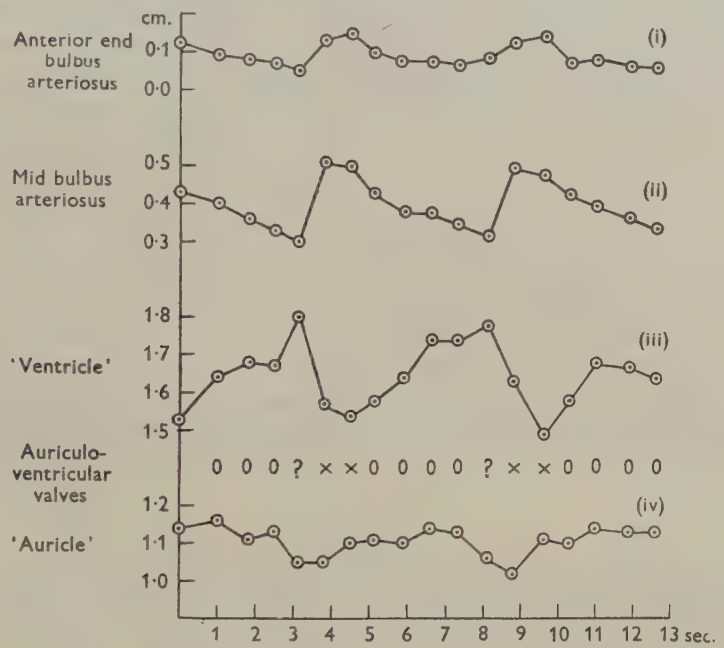
The time taken for the Thorotrast injected into the caudal vein to reach the ventricle was $4{\cdot}2 \pm 1{\cdot}8$ sec. in fifteen animals. This time is of no physiological significance, but it is of course of practical importance to know it.

On anatomical grounds, the easiest route from the caudal vein to the heart might well appear to be via the caudal vein—hepatic portal vein anastomosis and the hepatic portal system (Mott, 1950). In fact, however, in all but two of fifteen experiments, the bulk of the injection travelled via the renal portal system to the right posterior cardinal vein (which is much larger than the left) and thence to the heart.

The heart

The precise picture obtained of the heart naturally depends on the phase of contraction when the Thorotrast reaches it. If the sinu-auricular valves are closed, the Thorotrast is held up. If they are open, a varying amount will get through to the auricle and perhaps to the ventricle. Pl. 8, fig. 3, shows the ventricle in diastole containing a small amount of contrast medium, and the sinus well filled with the sinu-auricular valves obviously closed.

The auriculo-ventricular valves can be seen in the ventrodorsal projection, Pl. 8, fig. 7, and the jet of blood from the auricle entering the ventricle in Pl. 8, fig. 2. Emptying of the ventricle is shown in Pl. 8, fig. 4.



Text-fig. 2. Diagram showing changes with time in size of various parts of the heart shadow. Measurements made as indicated in Pl. 8, fig. 6, of: (i) diameter of extreme anterior end of bulbus arteriosus; (ii) diameter of bulbus arteriosus at mid-point; (iii) length of auricle and ventricle shadow, most of which is contributed by the ventricle; (iv) width of auricle and ventricle shadow, most of which is contributed by the auricle. 0 = auriculo-ventricular valves open; × = auriculo-ventricular valves shut.

The most remarkable feature noticed in connexion with the heart beat were the 'contractions' of the bulbus arteriosus, which were often very considerable, particularly at its anterior end. Plate 8, figs. 6, 7, shows two of a series of radiographs taken as fast as possible from which the dimensions of various parts of the heart have been measured and plotted in Text-fig. 2. The curve representing the greatest width of the bulbus arteriosus approximates to a mirror image of that representing the greatest length of the shadow of the auricle and ventricle (the greater part of which is contributed by the ventricle). This is a striking indication of the elasticity of the

bulbus arteriosus. In extreme cases the anterior end of the bulbus arteriosus appears to be able to shut down almost completely; it is possible that in this way it supplements the action of the ventriculo-bulbar valves (Pl. 8, fig. 6).

Passage of blood through the gills

Part of a typical series of radiographs from which the circulation times were estimated is shown in Pl. 9. The afferent branchial arteries fill in from 1 to 2 sec. after the ventricular contraction and before the next heart beat. Sometimes an appearance of streamlining (Pl. 9, fig. 2) can be seen in the ventral aorta. This presumably indicates incomplete mixing of blood from the right posterior cardinal and other veins entering the heart. The filling of the afferent branchials is not always quite simultaneous, and similarly with the efferent branchials. To get an estimate of the time of passage of the blood through the gills, the times have been recorded of the arrival of the injection at the two posterior afferent and two posterior efferent arteries respectively. In fourteen experiments the circulation time through the gills was $5{\cdot}6 \pm 1{\cdot}9$ sec.

The circulus cephalicus and cranial arteries

These arteries are not nearly so conspicuous in the living animal as post-mortem radiographs of specimens injected with 10 % bismuth carbonate suspension might lead one to expect. An examination of 'double prints' suggests that the blood coming up the second efferent branchial artery flows only caudally—the first two efferent branchial arteries contribute to the circulus cephalicus in the eel (Ridewood, 1899)—and it is probable that much of that coming up the first efferent branchial also flows backwards. Certainly all the emphasis is on the caudal flow, and the

Table 1

(Times (in seconds) taken by Thorotrast, introduced into the caudal vein, to pass from the efferent branchial arteries to other anterior arteries. The time at the efferent branchial arteries is arbitrarily taken as zero.) The number of experiments is given in brackets.

Coronary artery (cephalic, proximal)	$4{\cdot}2 \pm 1{\cdot}1$	(3)
Coronary artery (cephalic, distal)	$10{\cdot}0 \pm 4{\cdot}4$	(2)
Coronary artery (caudal)	$3{\cdot}9 \pm 1{\cdot}4$	(6)
Brachial artery	$3{\cdot}7 \pm 0{\cdot}7$	(8)
Cranial arteries	$8{\cdot}1 \pm 4{\cdot}4$	(5)
Orbital artery	$4{\cdot}6 \pm 1{\cdot}3$	(2)
Opthalmic artery	$5{\cdot}3 \pm 0{\cdot}9$	(3)
Retinal artery	$5{\cdot}9$	(1)
Anterior gut arteries	$3{\cdot}8 \pm 0{\cdot}7$	(8)

quantitity of blood made visible by the radiopaque injection in the head is very small indeed. Table 1 gives an indication of the times elapsing between the appearance of the blood in the posterior efferent branchial arteries and its appearance in various arteries in the anterior part of the body. It must be remembered that the smaller the artery, the greater is the possibility of missing its earliest filling on the radiograph, since there may not be a sufficient quantity of radiopaque material in it to cast a shadow.

The circulation through the viscera as shown by the injection of Thorotrast into the ventral aorta

After injection into the ventral aorta, the time taken for the radiopaque material to reach the level of the anterior gut arteries was exceedingly variable (2·9–16·6 sec.), and is probably of no significance. Only in one eel did the injection take a time (7·7 sec.) of the order expected from the previous experiments (injection into the caudal vein); otherwise it either tended to be rather short (2·9–3·6 sec.) which may mean the injection was made too rapidly, or it took 11–16 sec. This latter result was probably due to temporary inhibition of the heart beat following the injection, through the operation of a branchial depressor reflex which was discovered in later experiments (Mott, unpublished). The distribution of blood to the various parts of the gut and other viscera is on the whole slow (Pl. 10). The various arteries do not always fill in the same order, or at all, and it is not unusual for an artery to fill with radiopaque material, which then moves only slowly. Such 'rootless' branches of the dorsal aorta present rather a curious aspect when abandoned by the main bulk of the injection which has proceeded down the dorsal aorta. One would not, of course, expect the gut circulation in a starving animal to be particularly active, but it is evident that it is by no means non-existent, particularly in the region of the pylorus. Some circulation times, calculated from the arrival of the blood at the level of the anterior gut arteries in the dorsal aorta, are given in Table 2. These

Table 2

(Times (in seconds) elapsing between the appearance of Thorotrast, introduced into the ventral aorta, in the dorsal aorta at the level of the anterior gut arteries (taken as zero time) and some visceral arteries.)

Coeliac artery	8·4 ± 6·2	(9)
Lieno intestinal artery	10·5 ± 9·8	(10)
Pneumogastric artery (intestinal branch)	11·0 ± 10·4	(10)
Gas bladder artery	8·2 ± 9·6	(8)
Splenic artery	10·9 ± 8·5	(9)
Anterior mesenteric artery	17·3 ± 10·3	(10)
Anterior renal artery	19·7 ± 10·1	(10)
Meeting of mesenteric and lieno-intestinal arteries	45·9 ± 36·6	(6)

times are necessarily very rough, as the frames are separated by long intervals of up to 10 sec. and it is more than usually difficult to be consistent in interpreting radiographs of this region because the circulation is so very slow. In general, a vessel has been considered reached by the contrast medium when about two-thirds of its length is easily visible; thus though the splenic artery is a branch of the lieno-intestinal, the latter may not fill until after the former, according to these criteria. It is not unusual for the proximal parts of the anterior mesenteric and anterior renal arteries to fill and the radiopaque material to remain static, in which case the time taken is that at which no further progress seems to be made by the injection.

The pneumatic duct and gas gland circulation

There are two morphologically distinct routes by which blood entering the gas gland artery can return to the heart (Mott, 1950). These are the pneumatic duct

vein, which receives blood from the capillary network in the wall of the pneumatic duct and runs straight back to the heart; and the gas gland vein receiving blood from the red bodies which empties into the hepatic portal vein. It appears from the radiographic records that, as a rule, one or other of these circulations is in action, or, sometimes, both together. The relevant circulation times are given in Tables 2 and 3, calculated from the arrival of the injection at the level of the anterior gut arteries in the dorsal aorta.

The posterior venous return

The time which elapses before the radiopaque material appears in the veins is often very great, especially when the heart rate is slow. Some of these times are given in Table 3 calculated from the arrival of the blood at anterior gut artery level in the dorsal aorta.

Table 3

(Times (in seconds) elapsing between the appearance of Thorotrast, injected into the ventral aorta, in the dorsal aorta at the level of the anterior gut arteries and in the visceral veins.

Hepatic portal vein at the level of:		
Liver	16·8 ± 10·6	(9)
Spleen	11·8 ± 3·4	(5)
Mid-intestine	44·4 ± 35·0	(7)
Branches of the hepatic portal vein in liver	20·5 ± 15·9	(4)
Pneumatic duct vein	19·4 ± 14·9	(7)
Gas bladder vein	19·0 ± 7·8	(4)
Posterior end of posterior cardinal vein	61·1 ± 36·5	(3)

There is often an appearance of retrograde flow in the hepatic portal vein. Whether this is genuine or not is extremely difficult to ascertain, as the more anterior part of the vein naturally becomes opaque first, since the arteries whose contents contribute to its opacity are themselves opaque before those whose contents eventually appear in the posterior parts of the hepatic portal vein. In several eels, however, there was a definite indication of a forward flow in the posterior half of the hepatic portal vein whose shadow joined up with the shadow in the anterior part of the vessel. It seems likely, therefore, that any retrograde movements are only apparent.

The anterior venous return

No contrast medium has been detected in the anterior venous return so that all that can be stated is that it does not occur within about 40 sec. of the blood leaving the ventricle. So little blood seems to enter the cranial arteries, however, that it is quite possible that some time would have to elapse before any contrast medium became visible in the anterior veins. That the contrast medium does reach them is shown by the fact that they eventually become just as opaque as other parts of the venous system.

DISCUSSION

The general picture obtained of the behaviour of the eel's circulation from the serial radiographs described above does not differ markedly from what would be assumed from a knowledge of its morphology. There are, however, some subsidiary points of interest.

The bulbus arteriosus is well known to be composed of elastic tissue, but it does not appear to have been generally realized that its behaviour can be literally elastic, though von Skramlik (1935) described it as a 'windkessel'. It will help to spread out the rise of pressure produced by ventricular systole over a longer period of time than would otherwise be the case, and thus avoid subjection of the proximal ends of the gills to too extensive fluctuations in pressure as von Brucke (1852) suggested. Pressure curves in the bulbus arteriosus and ventral aorta were later recorded with a condenser manometer, amplifier and cathode-ray oscilloscope. There was considerable flattening of the peak pressure recorded in the ventral aorta compared with that in the bulbus arteriosus. The functional analogy with the aortic arch of mammals is obvious.

It is clear that the gills offer a considerable resistance to the passage of the blood though not perhaps as much as might have been expected. The various parts of the heart-gill complex, should not, however, be considered separately. The pressure developed in the ventral aorta and afferent branchials must depend on the resistance of the gills as well as on the force of the heart beat and the resultant of both these factors may be reflected in the behaviour of the bulbus arteriosus. It is possible that complete mixing of the venous blood from various parts of the body does not occur in the ventricle, since streamlines were observed on occasion in the ventral aorta.

The amount of blood reaching the head seems small in view of the great importance always attached to this part of the circulation. It must be remembered, however, that the brain of a fish only forms a very small part of its body weight. Even so, this part of the circulation is less in evidence than one might expect from its morphology. All the emphasis is on the caudal flow of blood from the gills, certainly from the second, third, and fourth gill arches.

Interest in the visceral circulation chiefly attaches to that part of it supplying the pneumatic duct and gas glands, and to the fact that there appear to be two alternative circulations here. From what is known of the function of the gas glands and the homologues of the pneumatic duct in fish, it is tempting to associate activity in the pneumatic duct circulation with gas absorption and that in the red body circulation with gas secretion. This hypothesis is perhaps supported by the fact that the latter tends to be more in evidence when the duration of the experiment has been less. The blood in the pneumatic duct vein of the anaesthetized fish often appears very red, as if well oxygenated.

The circulation times in the anaesthetized eel are obviously subject to considerable variation and all are slow when compared with those known for higher Vertebrates. On the other hand, it must be remembered that these observations have been recorded in anaesthetized eels, which are naturally quiescent. The venous return is slow under these conditions, and it may well be that the active eel maintains a more rapid venous return through the effect of its continuous co-ordinated bodily undulations on the veins.

SUMMARY

1. A description is given of an angiographic method for studying the circulation of blood in the anaesthetized common eel.

2. The valves of the heart are concluded to be competent.

3. The extreme elasticity of the bulbus arteriosus is noted, especially of its anterior end.

4. The circulation time through the gills was $5{\cdot}6 \pm 1{\cdot}9$ sec.

5. The circulation in the head is relatively inconspicuous; all the emphasis is on the backward flow of blood from the gills.

6. The circulation times to the viscera are very variable.

7. It appears that the two alternative routes of venous return from the pneumatic duct artery, which are the pneumatic duct vein and the gas gland vein, may both be operating together or either may operate alone.

My thanks are due to Dr G. S. Dawes for helpful criticism, to the late Dr A. E. Barclay who introduced me to the techniques of cineradiography, and to Mr Maurice Tuckey for technical assistance. This work was carried out during the tenure of a D.S.I.R. maintenance grant.

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EXPLANATION OF PLATES

All the plates are made from contact prints of the original radiographs.

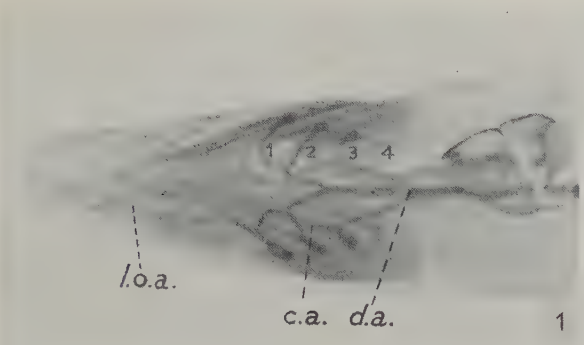
PLATE 8

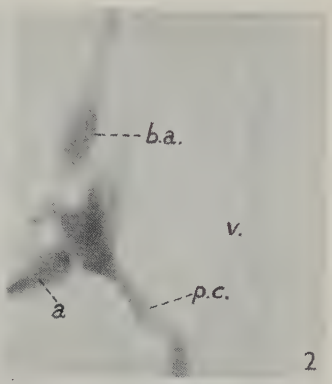
Fig. 1. Eel 55, run 1, dorsoventral projection: a positive of frame taken 6·1 sec. after injection of 1 ml. Thorotrast into caudal vein printed together with frame taken 14·4 sec. after injection. Note virtual elimination of skull and afferent branchial system; 1, 2, 3, 4, efferent branchial arteries; *l.o.a.*, left orbital artery; *d.a.*, dorsal aorta; *c.a.*, root of left cephalic coronary artery.

Fig. 2. Eel 25, run 2 (1st run failed), lateral projection 6·3 sec. after 4 ml. Thorotrast injected per caudal vein. Shows jet of blood entering ventricle from auricle, *a.*; bulbus arteriosus, *b.a.*, filled at previous heart beat; *p.c.*, right posterior cardinal vein; *v.*, vertebral column.

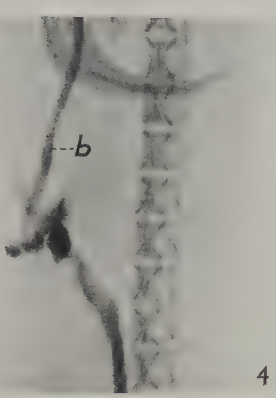
Figs. 3–5. Eel 38, run 1, lateral projection, three consecutive frames 3·9, 5·6 and 7·3 sec. respectively after injection of 1 ml. Thorotrast into caudal vein. Heart rate 50·5 per min. Fig. 3 shows ventricle in diastole and accumulation of ThO_2 in sinus venosus; Fig. 4 shows ventricle in systole and auricle well filled; Fig. 5 shows ventricle in diastole and ventriculo-bulbar valves shut, *s.*, sinus venosus; *v.*, ventricle; *b.*, bulbus arteriosus; *v.b.*, ventriculo-bulbar valves; *c.*, cleithrum; *a.*, afferent branchial artery.

Figs. 6, 7. Eel 52, run 2 B. Two consecutive frames from series from which Text-fig. 2 was constructed, ventrodorsal projection. Lines indicate distances measured. Heart rate 15 per min. *a.v.*, auriculo-ventricular valves.



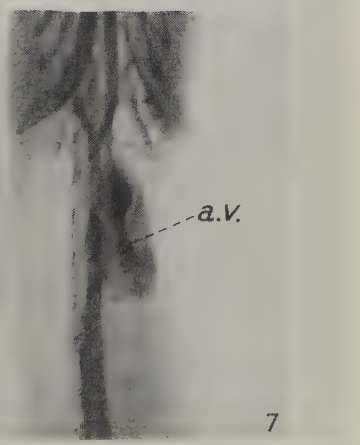




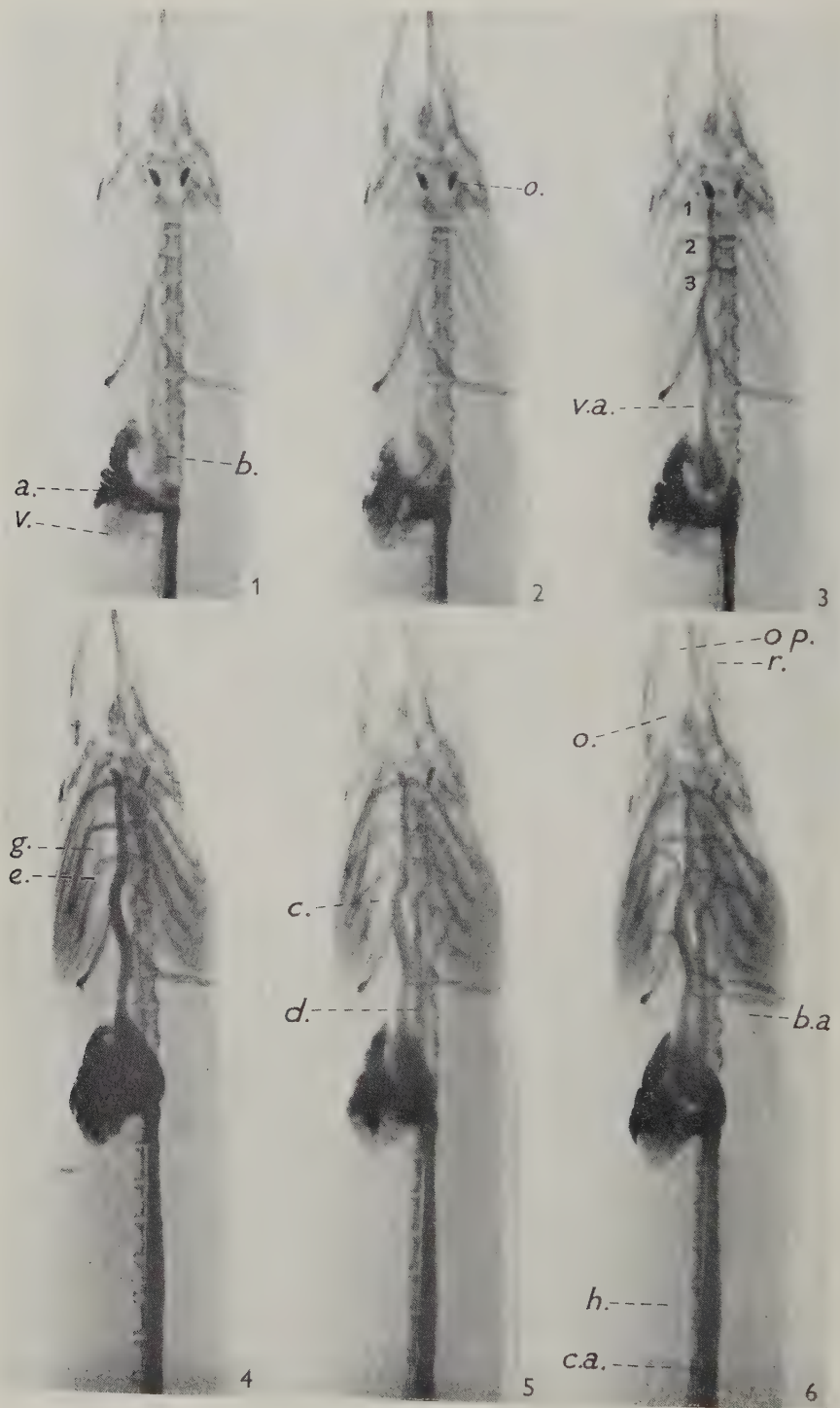




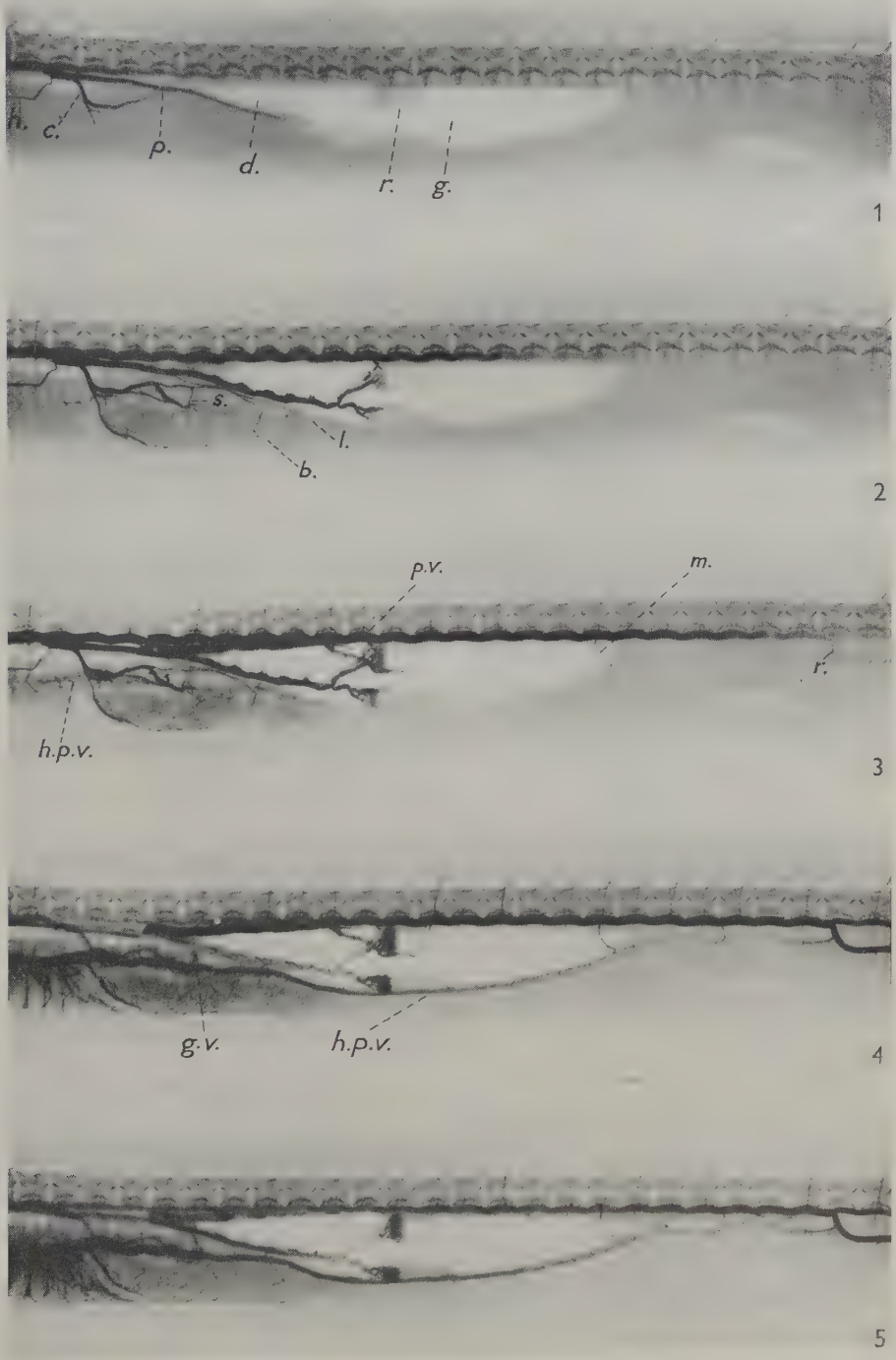




MOTT—RADIOLOGICAL OBSERVATIONS ON CARDIOVASCULAR SYSTEM IN *ANGUILLA ANGUILLA*



MOTT—RADIOLOGICAL OBSERVATIONS ON CARDIOVASCULAR SYSTEM IN *ANGUILLA ANGUILLA*



MOTT—RADIOLOGICAL OBSERVATIONS ON CARDIOVASCULAR SYSTEM IN *ANGUILLA ANGUILLA*

PLATE 9

Eel 55, run 1, dorsoventral projection. Six frames taken respectively 1·6, 2·4, 3·1, 8·3, 14·4 and 17·5 sec. after an injection of 1 ml. Thorotrast into the caudal vein. Heart rate 32 per min. *a.*, auricle; *b.*, bulbus arteriosus; *v.a.*, ventral aorta; *v.*, ventricle; *o.*, otolith; 1, 2, 3, afferent branchial arteries; *g.*, gill filaments; *e.*, efferent branchial artery; *d.*, dorsal aorta; *c.*, origin of cephalic coronary artery; *b.a.*, brachial artery; *h.* hepatic artery; *c.a.* coeliac artery; *r*, retina outlined by vessels; *o.*, orbital artery; *op.* opthalmic artery. Note in frame 2, jet of blood from ventricle entering bulbus arteriosus.

PLATE 10

Eel. 72, run 1. Five frames taken respectively 3, 6, 12, 24 and 39 sec. after the injection of 1 ml. Thorotrast into the ventral aorta. Heart rate 62 per min. *h.*, hepatic artery; *c.*, coeliac artery; *p.*, pneumogastric artery; *d.*, pneumatic duct; *g.*, gas bladder; *r.* (frame 1), red body; *l.*, lieno-intestinal artery; *s.*, splenic artery; *b.*, branch of pneumogastric artery; *p.v.* pneumatic duct vein; *m.*, mesenteric artery; *r.* (frame 3), anterior renal artery; *h.p.v.*, hepatic portal vein; *g.v.*, gas bladder vein.

A NEW MICRO-RESPIROMETER WITH AUTOMATIC SETTING AND RECORDING APPARATUS

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(With Plate 11 and Ten Text-figures)

The instrument was designed to measure the O_2 uptake of living material which consumes between 0·01 and 5·0 μl. O_2/ hr. Its accuracy is $\pm$0·001 μl., and it has the following advantages over other micro-respirometers of similar sensitivity.

(*a*) It is robust, easy to handle and is suitable for routine use.

(*b*) The respirometer constant is independent of the gas volume of the reaction vessel.

(*c*) Its sensitivity is independent of the volume of the reaction vessel, and it can therefore be used with relatively large amounts of living material.

(*d*) Experiments can be carried out over a wide range of temperatures without appreciably altering the accuracy.

(*e*) It can be coupled to apparatus (described at the end of this paper), which automatically measures and records the rate of O_2 uptake.

A comprehensive review of the better known micro-respirometers has been given by Tobias (1943), but to facilitate comparison with the present instrument a list of the characteristics of some of them are given in Table 1.

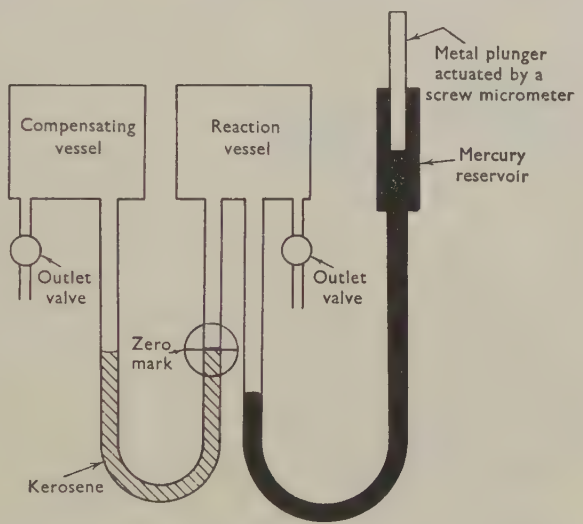
Table 1. *The characteristics of different types of micro-respirometer*

	Sensitivity (smallest rate that can be measured) (μl./hr.)	Accuracy (μl.)	Volume of the reaction vessel (μl.)
Barcroft Warburg	10	$\pm$0·5	5000
Steffanelli (1937)	0·06	$\pm$0·003	30–100
Cartesian diver			
(1) Linderstrøm-Lang & Holter (1943)	0·01	$\pm$0·0002	10
(2) Zeuthen (1943)	0·002	$\pm$0·00002	0·1
The new instrument	0·01	$\pm$0·001	50–100

PRINCIPLE

The principle of the instrument can best be followed from the diagram (Text-fig. 1). It consists essentially of two vessels—a reaction vessel and a compensating vessel—connected by a U-tube containing a drop of kerosene. The volume of the reaction vessel can be adjusted at will by means of a pipette. This consists of a mercury-filled reservoir from which known volumes of mercury can be displaced by means

of a metal plunger actuated by a screw micrometer (the latter is not shown in Text-fig. 1). When the two vessels are cut off from the outside atmosphere, any pressure change in one relative to the other will be followed by a corresponding movement of the kerosene droplet. In order to determine the amount of gas absorbed or evolved by the experimental material, the two vessels are set up so that they differ only in the single respect that one—the reaction vessel—contains the material to be studied. The respirometer is placed in a water-bath, and after a suitable equilibration period the outlet valves are closed. After a short time the movement of the kerosene will represent changes in pressure due to the experimental material. The volume of the reaction vessel is then adjusted with the pipette until the kerosene meniscus is



Text-fig. 1. Diagram to show principle of the micro-respirometer.

brought level with a fixed point on the U-tube. After a known interval the meniscus is again adjusted to the same point; the gas pressure of the whole system is thereby restored to its original value. The volume of the length of plunger introduced or withdrawn from the reservoir is equal to the volume of gas absorbed or evolved in the reaction vessel, at the temperature and pressure of the experiment.

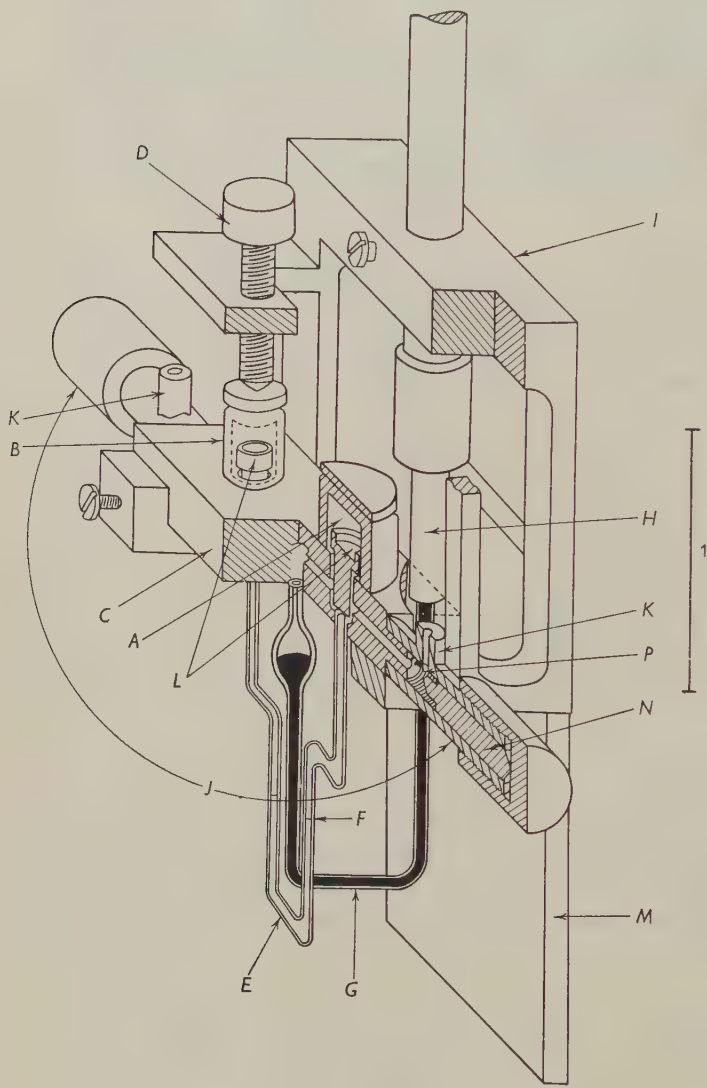
GENERAL DESCRIPTION OF THE MICRO-RESPIROMETER

The manometer component (Text-fig. 2) and the pipette (Text-fig. 3) are described separately. The assembled instrument is shown in Pl. 11, fig. 2.

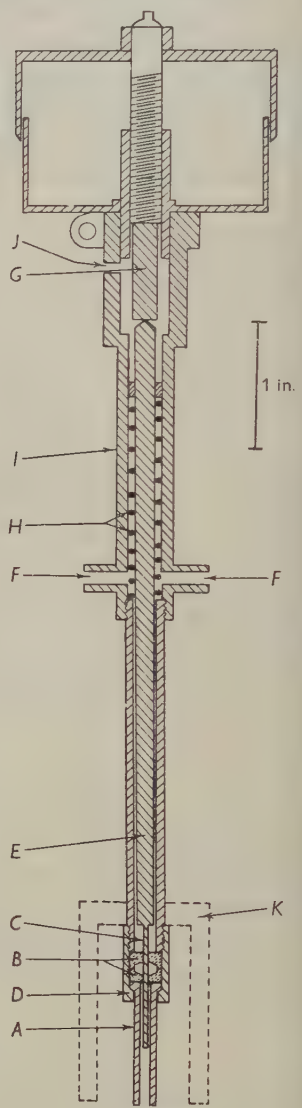
(i) *The manometer component* (Text-fig. 2)

The reaction vessel and the compensating vessel (Text-fig. 2*A*) are both detachable, and consist of small glass cups, with ground edges (which are greased). Both vessels are clamped on to a stainless steel block (Text-fig. 2*C*) by means of two thumb screws (Text-fig. 2*D*). Sealed into the block and opening into the cavity

under each vessel, is a small, fine-bore capillary U-tube (Text-fig. 2*E*), containing a drop of kerosene. The meniscus of the kerosene (Text-fig. 2*F*) is observed through a low-powered microscope (Text-fig. 4). A glass capillary (Text-fig. 2*G*), of slightly larger bore, connects the reaction vessel and the reservoir of the pipette (Text-fig. 2*H*).



Text-fig. 2.



Text-fig. 3.

Text-fig. 2. The manometer component. *A*, reaction vessel; *B*, compensating vessel; *C*, stainless steel block; *D*, clamping screw; *E*, U-tube; *F*, kerosene meniscus; *G*, pipette capillary; *H*, mercury reservoir; *I*, metal frame; *J*, needle valves; *K*, nipple for outlet tube; *L*, recessed metal plugs for experimental material; *M*, Perspex plate; *N*, needle; *P*, spring.

Text-fig. 3. The pipette component. *A*, mercury reservoir; *B*, vulcanized rubber washers, with recess for Hg seal; *C*, silver steel plunger; *D*, screw collar; *E*, push rod; *F*, nipples for outlet tubes; *G*, micrometer; *H*, spring; *I*, pipette casing; *J*, outlet; *K*, position of metal frame to hold manometer.

The metal block is clamped on to a small metal frame (Text-fig. 2*I*), attached to the casing of the pipette. Both vessels communicate with the outside atmosphere through holes in the block which can be closed by needle valves (Text-fig. 2*J*). The latter are connected to the pipette casing by rubber tubes (Pl. 11, fig. 2). Small plugs of metal or glass (Text-fig. 2*L*), with recessed ends fit loosely into the cavities under each vessel. The capillaries are protected by a sheet of Perspex (Text-fig. 2*M*).

(ii) *The pipette* (Text-fig. 3)

The pipette consists of a small mercury reservoir made of stainless steel (Text-fig. 3*A*), closed at one end by two recessed vulcanized rubber washers (Text-fig. 3*B*), enclosing a drop of mercury, through which passes a silver steel plunger of known diameter (Text-fig. 3*C*). The reservoir and washers are held in place by a collar (Text-fig. 3*D*). The plunger is attached to the end of a push rod (Text-fig. 3*E*), held in contact with the shank of the micrometer (Text-fig. 3*G*), by a spring (Text-fig. 3*H*). The rubber tubes leading from the needle valves on the metal block are connected to two nipples on the casing (Text-fig. 3*F*) which opens to the atmosphere through a hole near the top (Text-fig. 3*J*).

ACCESSORY APPARATUS

The general arrangement of the apparatus is shown in Text-fig. 4 and Pl. 11, fig. 1. The respirometers are clamped on to the lower bar of a supporting frame so that the manometer component is completely submerged in the water. The microscope is so arranged that it can be moved along the frame and focused on each of the U-tubes in turn. The latter are illuminated by small 12 V. lamps, the light from which passes through curved Perspex rods, so that the lamps can be placed well away from the surface of the water-bath. The water-bath has a temperature differential of approximately $\pm 0{\cdot}03^{\circ}$ C.

NOTES ON THE DESIGN

(1) *The metal block and reaction vessel*

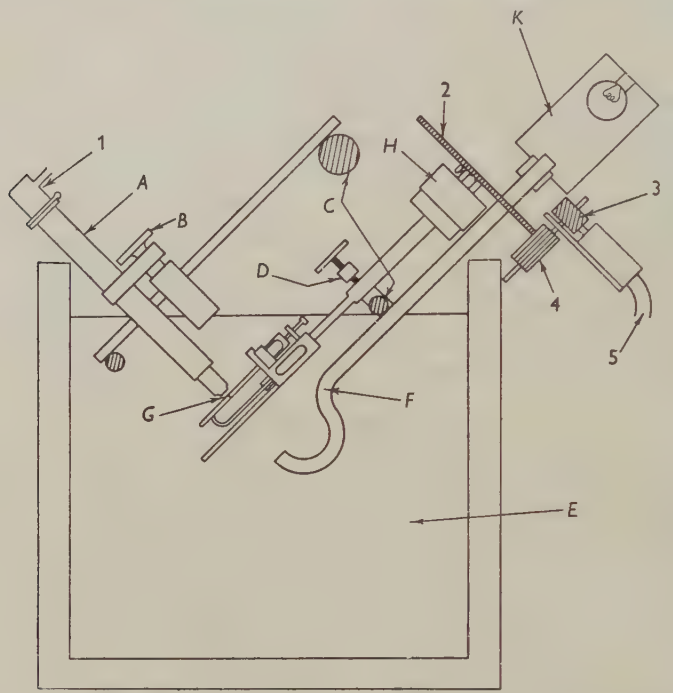
The reaction vessel and compensating vessel are placed as close together as possible in order to avoid temperature differences between them. The metal block, besides providing a rigid base for the vessels, tends to smooth out temperature variations of short duration.

The vessels can be of any size, but the diameter of the base in contact with the metal block must be as small as possible. When the diameter is large, small movements of the vessel relative to the block, which occur when the grease seal is gradually expressed, give rise to oscillations of the manometer fluid. For general use, 4 mm. is a convenient diameter for the base of the vessel; should larger vessels be required, they must be made with bulging, rather than parallel sides.

(2) *Capillaries*

The bore of the capillary U-tube is between 0·1 and 0·2 mm. Theoretically, a finer bore would increase the sensitivity but, in practice, it is found that even the best manometer fluids tend to stick and move erratically in capillaries of less than 0·1 mm.

unless they are frequently cleaned. The use of a microscope with two-third objective and ×8 ocular makes it possible to adjust the meniscus to within 0·01 mm. of a given position. This represents a volume change of about 0·0006 μl. in a 0·1 mm. diameter capillary which is less than the smallest volume that can be measured with the pipette.



Text-fig. 4. Diagram of a cross-section of the water-bath to show arrangement of the respirometers and accessory apparatus. *A*, microscope; *B*, focusing screw; *C*, supporting frame carrying microscope and respirometers; *D*, clamp to hold respirometer; *E*, water-bath; *F*, Perspex light duct; *G*, kerosene meniscus in manometer U-tube; *H*, micrometer head; *K*, lamp housing (this is replaced by a more powerful lamp and revolving shutter for automatic recording). Accessory apparatus used for setting the respirometer automatically. 1, hinged housing for photocell; 2, gear wheel; 3, worm gear attached to driving shaft; 4, gear wheel; 5, flexible drive from the setting motor.

(3) *Zero mark*

A scratch on a very small droplet of black wax is the most satisfactory mark; engraved marks weaken the capillaries.

(4) *Manometer fluid*

The most satisfactory manometer fluid is a light kerosene marketed under the name of 'odourless distillate' which has a low viscosity and does not evaporate easily. Petroleum ether is too volatile.

(5) *Needle valves*

The needle valves require careful construction. The needle (Text-fig. 2*N*) fits loosely in the sleeve, and it is important that when it makes contact with its seating

it should come to rest. (The needles are allowed to make their own seating and are not ground in.) Before the instrument is assembled the valves are tested with a vacuum pump. A properly constructed valve should hold a vacuum of 3 mm. mercury for over an hour, but it is sometimes necessary to grease the needle lightly before it will pass this test.

(6) *Pipette*

The pipette is the central support of the instrument. It is larger than the rest of the respirometer, but only the mercury contained in the reservoir is liable to give rise to errors when the temperature varies. These errors, however, are not important if the volume of mercury does not exceed 10 μl. No error is introduced by the thermal expansion of the metal push rod because it is compensated by a corresponding expansion of the metal casing of the pipette.

(7) *Pipette plunger*

The terminal half inch of the plunger, which is used to displace mercury in the reservoir, must have a uniform diameter. The smallest diameter that can be used conveniently is $\frac{1}{32}$ in. because smaller plungers bend and give rise to inaccuracies. The accuracy of the pipette depends on the accuracy with which the plunger displaces the mercury, for this reason the mercury in the reservoir must be free of air bubbles. Their presence can be detected by applying a vacuum to the end of the pipette capillary and observing the Hg meniscus.

EXPERIMENTAL PROCEDURE

(1) *Equilibration*

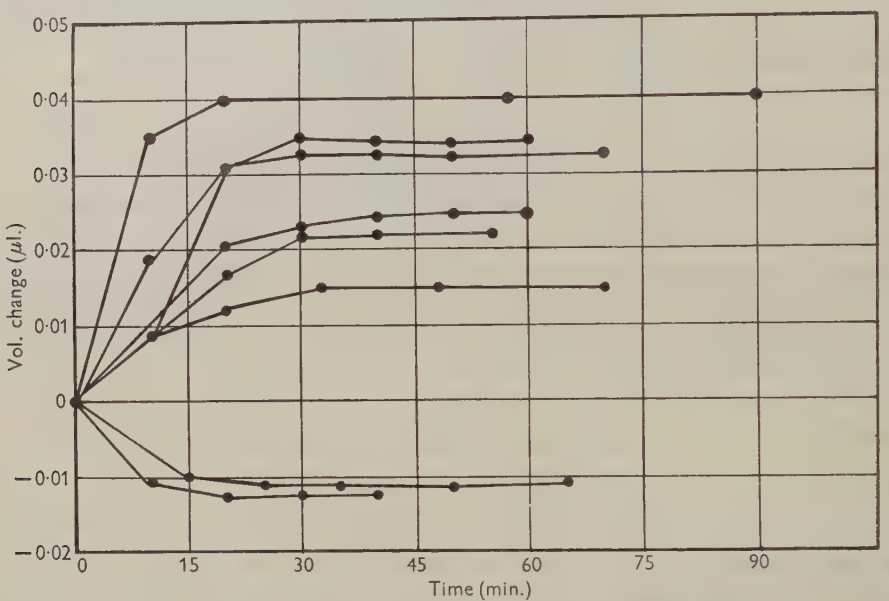
The time that must be allowed for the instruments to equilibrate depends on the difference between the bath and room temperature. In order to determine the length of equilibration period required, the instruments are set up with both chambers empty. The valves are closed as soon as they have been placed in the water-bath, and the volume changes are measured. A series of typical equilibration curves are shown in Text-fig. 5. An equilibration period of half an hour is adequate for a temperature difference of 8° C.; this can be reduced to 20 min. for a temperature difference of 1°.

(2) *The measurement of* O_2 *uptake*

The reaction vessel may be set up in one of two ways depending on the type of experimental material. Solid objects, such as insect eggs, are placed in the recessed end of the metal plug (Text-fig. 2*L*); suspensions and material in a liquid medium are placed in a hanging drop in the roof of the reaction chamber. The CO_2 absorbent (M/10 KOH) is placed on a disk of no. 40 Whatman filter-paper either in the top of the chamber or on the recessed plug. When the O_2 uptake of suspensions is being measured, the volume of the drop must be chosen carefully. There is no stirring in these instruments, and therefore the volume of the drop in relation to its surface must be such that the rate of diffusion of O_2 does not become a limiting factor.

After they have been filled, four small drops of 'Lubriseal' grease are placed on

the ground surfaces of the vessels, and they are then clamped on to the metal block with the thumb screws. The instrument is placed in the water-bath. The rubber tubes attached to the needle valves are gently squeezed to make sure the capillaries are not blocked. After an appropriate equilibration period, the valves are closed and tested by bending and squeezing the rubber tubes. Five minutes later the meniscus is adjusted to the zero point on the U-tube. This adjustment is repeated at intervals. The actual volume changes are obtained by multiplying the difference between successive readings of the micrometer by the volume per unit length of the plunger. A series of respiration experiments are described in Appendix I.



Text-fig. 5. A series of equilibration curves. Room temperature, 17° C., bath temperature, 25° C.

(3) *Gas mixtures*

The vessels can be filled with any desired gas mixture, but the plugs which normally hold the experimental material cannot be used. If a holder is required it must have a hole down the centre so that the gas mixture can reach the upper part of the chamber.

The vessels are greased and placed in position but are not clamped down; so that the gas can escape between the droplets of grease. The gas supply is connected to the rubber tube attached to the needle valve.

When the gas has been turned on the kerosene droplet is observed to ensure that an adequate pressure is maintained in the vessel, and to assist ventilation the micrometer is screwed up and down at intervals. The gas supply is then disconnected; the chambers are clamped down on to the block and the needle valves are closed.

(4) *Mixing reagents during an experiment*

The inner surface of the reaction vessel is either waxed or covered with a thin layer of 'Silicone' grease. The reagent with the larger volume is placed in the top of the vessel, and the other is placed in a droplet on the side. A small iron-filled glass bead is placed in the latter and the reagents are mixed by drawing the two droplets together by means of a magnet.

THE AUTOMATIC SETTING AND RECORDING APPARATUS

The apparatus is designed to interfere as little as possible with the normal arrangement of the respirometer. The only addition to the instrument itself is a large gear wheel attached to the micrometer head (Text-fig. 4, 2) which is coupled by a system of gears, and a flexible drive to an electric motor by means of which the micrometer is adjusted. The motor is controlled by a photocell attached to the microscope.

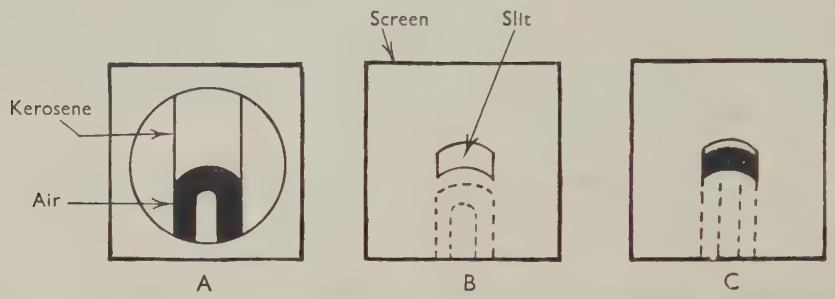
The microscope is focused on the capillary U-tube in the usual way, but the eyepiece is replaced by an opaque screen, in which is a slit cut to fit the image of the kerosene meniscus (Text-fig. 6B). The photocell, carried in a hinged housing so that it can be swung into place without disturbing the microscope (Text-fig. 4, 1), is illuminated by light passing through the slit in the screen. The amplifier connected to the photocell is so arranged that as long as the field of the slit is occupied by kerosene (Text-fig. 6B) sufficient current is passed by the cell to operate the relay which switches on the electric motor coupled to the micrometer, but as soon as the meniscus enters the field and reduces the light intensity below a certain level (Text-fig. 6C) the relay opens and the motor is switched off. In this way the device is able to set the meniscus to a fixed point whenever it is switched on. The present instrument uses an intermittent light source and an a.c. amplifier (as described in Appendix III) and is able to set the meniscus as accurately as it can be set by hand.

In order to get a sufficiently high intensity of light at the cathode of the photocell, the standard curved Perspex rod is replaced by a straight one with convex ends and a small mirror to reflect the light up on to the U-tube. In this way a strong beam of light from a 12 V. ribbon filament projector lamp is focused on the capillary.

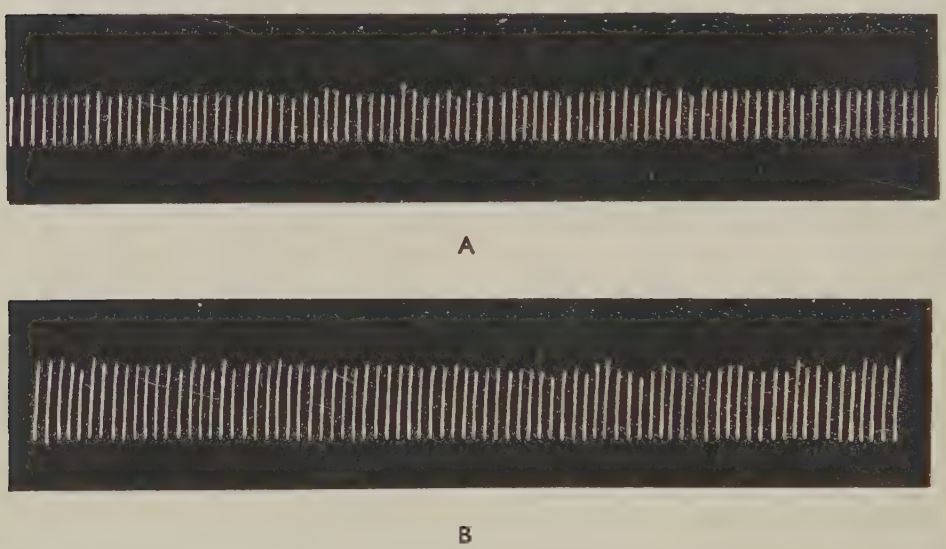
The device records intermittently. The amplifier circuit and light source are switched on by a time switch at intervals varying from one to six times per hour. If the volume of gas in the reaction chamber has decreased in the interval, then the meniscus will have moved up past the control slit so that enough light will fall on the photocell to operate the motor switch; the motor then screws the micrometer head down until the image of the meniscus reaches its zero position. The relay then opens and the motor stops.

The number of revolutions of the motor required to restore the meniscus to its zero position each time the amplifier and light are switched on is recorded automatically. In the early instruments the record was a series of vertical lines on the smoked drum of a kymograph, the interval between each being the interval between successive settings of the meniscus, the height representing the number of revolutions of the motor and hence the amount of oxygen consumed in each interval. A group

of recordings are shown in Text-fig. 7. This method of recording has now been replaced by a revolution counter which prints the number of revolutions on a paper strip.



Text-fig. 6. A, appearance of the meniscus in the eyepiece of the microscope. B, the screen with the control slit. When the meniscus is in the position shown by the dotted line, there is sufficient light falling on the cathode of the photocell to operate the relay controlling the setting motor. C, the zero position of the meniscus. In this position insufficient light falls on the cell to operate the relay and the setting motor stops.



Text-fig. 7. The O_2 uptake of single newt eggs (*Triturus alpestris*) recorded by the automatic respirometer. The height of each line represents the amount of the O_2 consumed. The space between each line represents an interval of 10 min. Recording A, O_2 uptake of an unfertilized egg; B, O_2 uptake of a fertilized egg, from 8 to 20 hr. at 20° C. (Records are read from right to left.)

SUMMARY

A new micro-respirometer is described, capable of measuring rates of volume change from 5 μl./hr. down to 0·01 μl./hr. to the nearest 0·001 μl. It is a constant-pressure nul-reading instrument and the actual volumes of gas absorbed or evolved are obtained from readings of the instrument by a simple multiplication.

A device is described which sets the instrument automatically and records the volume changes at regular intervals.

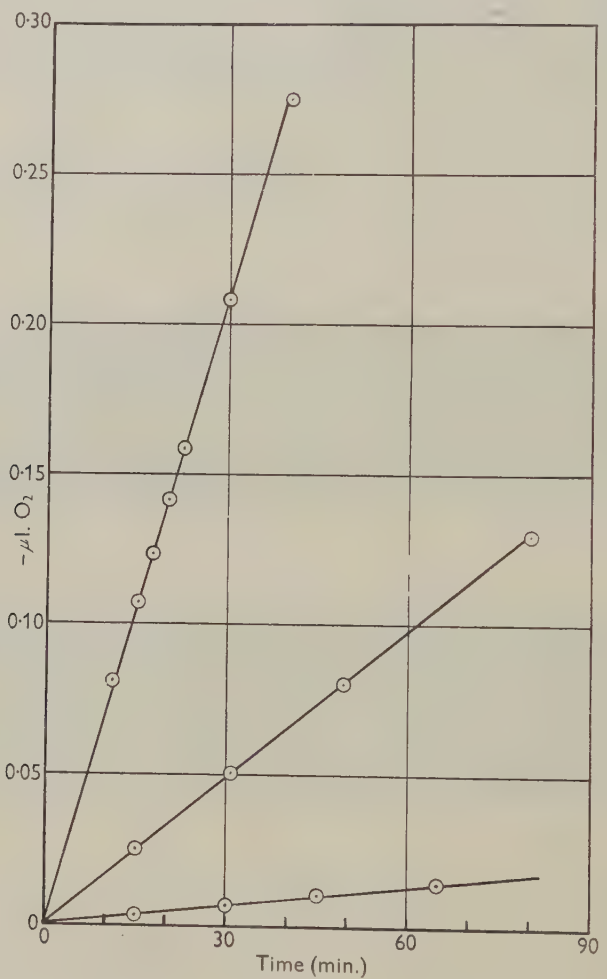
I wish to thank Prof. J. Gray, F.R.S., in whose department the instrument was developed, and also all those members of the staff who have helped me with the design and construction of the instrument, in particular Dr Ralph Brown. I should also like to thank Mr S. Falloon of the Cavendish Laboratory for designing the amplifier which has made the automatic attachment possible, and Lord Rothschild for helpful criticism of the manuscript.

The instrument was designed while I was receiving a research grant from the Agricultural Research Council, and The Royal Society kindly made a grant for the construction of the automatic attachment.

APPENDIX I

Experimental results

The stability of the new micro-respirometers is demonstrated by the results of three experiments on the O_2 uptake of *Rhodnius prolixus* eggs, shown in Text-fig. 8, in which the O_2 uptake calculated from successive readings of the micrometer are plotted cumulatively.



Text-fig. 8. The results of three experiments plotted cumulatively to show the consistency of successive readings of the respirometer.

The consistency of individual instruments is illustrated by a series of controls from experiments on the O_2 uptake of the eggs of *R. prolixus*. The O_2 uptake of each batch of eggs was measured twice in the same respirometer. The determinations lasted 1 hr., and the eggs were removed from the respirometers between each.

Table 2. *Untreated controls from a series of experiments on the* O_2 *uptake of* Rhodnius prolixus *egg*

(Each sample was removed from the respirometer for 10 min. between the first and second determinations.)

Exp. no.		1	2	3	5	6	7
O_2 uptake (μl./egg/hr.)	1	0·041	0·056	0·083	0·150	0·146	0·197
	2	0·040	0·057	0·083	0·147	0·150	0·197
Difference		0·001	0·001	0·000	0·003	0·004	0·000

The results obtained for the rate of O_2 uptake of yeast suspensions by two of the new micro-respirometers and a set of standard Warburg manometers are shown in Table 3.

Table 3. *The* O_2 *uptake of yeast suspensions measured with the new micro-respirometers and standard Warburg manometers*

(The yeast suspension contained approx. 0·5 g. bakers' yeast + 100 c.c. M/50 phosphate buffer + 0·5 g. dextrose. Temperature of the experiment was 25° C.)

Exp.	Duration of exp. (min.)	New micro-respirometers Constants (1) 1·20 (2) 0·98 Vol. of suspension in reaction vessel, 5 μl. Rate of O_2 uptake in μl./5 μl./hr. (in brackets μl./ml./hr.)	Warburg manometers K (1) 1·066 O_2 (2) 1·202 Vol. of suspension in reaction vessel, 2 ml. Rate of O_2 uptake in μl./ml./hr.
1	45	(1) 0·395 (79·0) (2) 0·398 (79·6)	(1) 77·0 (2) 76·0
2	30	(1) 0·334 (66·8) (2) 0·340 (68·0)	(1) 67·0 (2) 66·0
3	50	(1) 0·342 (68·4) (2) 0·344 (68·8)	(1) 65·0 (2) 68·0
4	40	(1) 0·404 (80·8) (2) 0·392 (78·4)	(1) 79·0 (2) 81·0

The 5 μl. samples were measured with a Linderstrøm-Lang type-constriction pipette. Pipetting errors probably account for the variations in these experiments.

APPENDIX II

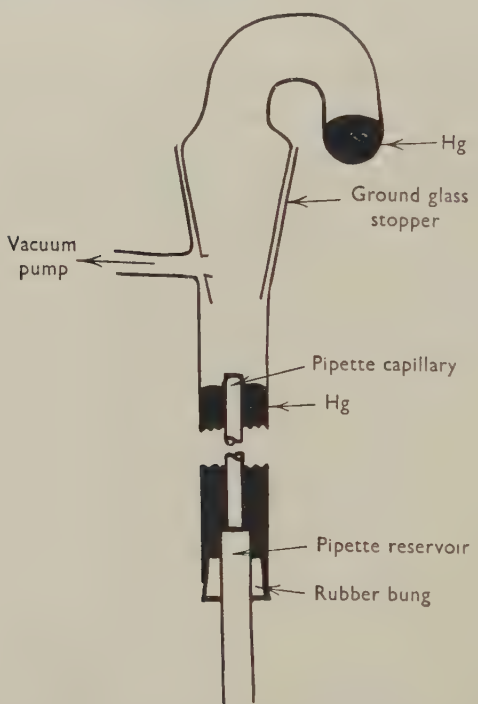
Assembling the apparatus

I. *Filling pipette*

(1) Measure diameter of plunger (taking care not to bend it). Examine under binocular microscope to see that there are no sharp edges that might scratch the vulcanite washers.

(2) Place vulcanite washers on plunger. Lightly grease with Lubriseal and place a small drop of Hg in the recess. Place reservoir over plunger. Screw down collar.

(3) Take a suitable length of capillary and fit into end of reservoir. Warm and place Apiezon wax 'W' on joint. Allow to cool.



Text-fig. 9. Apparatus used for filling the mercury reservoir of the pipette, consisting of a Thunberg tube with the bottom removed and fitted with a rubber bung to hold the mercury reservoir.

(4) Place Hg filler over reservoir and capillary and fill with Hg as shown (Text-fig. 9). Evacuate. Test to see that there is no leak in reservoir. Then cover end of capillary with Hg from side arm and remove from vacuum pump. The Hg will then be forced into the reservoir.

(5) Test again by turning on pump and observe mercury meniscus in capillary.

(6) Remove from filler and screw down micrometer to expel the Hg from the capillary, until, when the plunger is again withdrawn, the mercury can just be seen at the joint between the glass and the reservoir.

II. *Assembling manometer component*

(1) Slide the manometer frame into position and clamp the stainless steel block on to it.

(2) Bend the pipette capillary with a microflame until open end drops into the centre hole in stainless steel block.

(3) Remove the stainless steel block.

(4) Warm the ends of the capillary U-tube and melt on a drop of Apiezon 'W'. Do the same to the open end of the pipette capillary.

(5) Replace the block and heat it with the flame until the wax on the pipette capillary begins to flow over the metal. Then place, the U-tube in position. Heat the block until wax 'melts' on to the surface.

(6) Allow to cool. Place Perspex guard in position, and fit rubber tubing to nipples on the valves and pipette casing.

(7) With a bent capillary place a drop of odourless kerosene in the U-tube which should have been carefully cleaned before assembling.

Inspection before use

(1) Screw micrometer up and down and ensure that the mercury is moving smoothly.

(2) Examine the valve seatings to see that the edges of the holes are not scratched.

(3) Examine pointed ends of the needles. Warm in a flame and grease lightly.

(4) Test ground surfaces of vessels. A small drop of water sufficient to cover the ground surface when it is pressed on to the slide should be enough to make the vessel stick to the slide.

(5) Tilt instrument and observe kerosene to ensure that it moves freely.

Caution

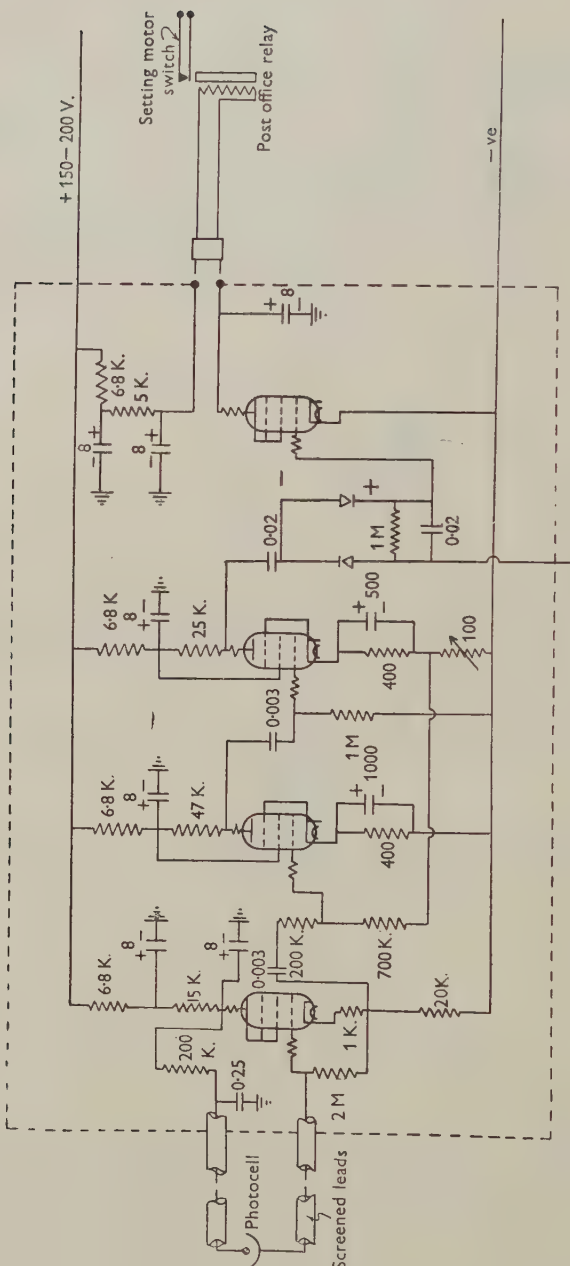
Care should be taken not to allow kerosene to touch the waxed joints. A small roll of filter-paper in the cavities in the block when adding kerosene is a useful safeguard.

APPENDIX III

The Amplifier

The circuit shown in Text-fig. 10 forms the link in the servo mechanism which controls the setting motor.

To avoid difficulties of d.c. amplifier operation, the light source is chopped at 500 c.p.s. and is focused as described on to the photocell ('Cintel' V.A. 31 made by Messrs Cine Television Ltd.)—a vacuum-type photocell with a sensitivity of about 10 μA/lumen. This is followed by a cathode follower stage with an effective input impedance of 25–30 MΩ. The cathode follower output is fed to a two-stage amplifier with a maximum gain of 10,000: gain is controlled by a variable negative feedback. The output from the amplifier stage at 500 c.p.s. is rectified in a voltage doubler circuit. The positive going d.c. output from the rectifier is connected directly to the



Text-fig. 10. Circuit diagram of amplifier.

(*Note.* All grid and anode pins, 200 Ω stoppers. Values of resistances in ohms. Values of condensers in micro-farads.)

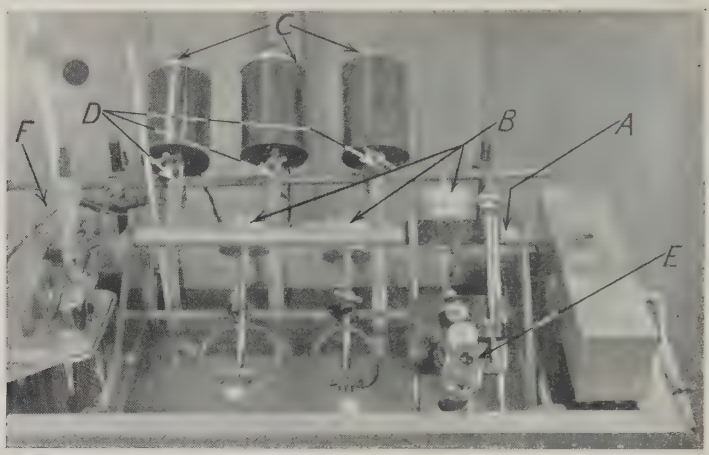


Fig. 1.

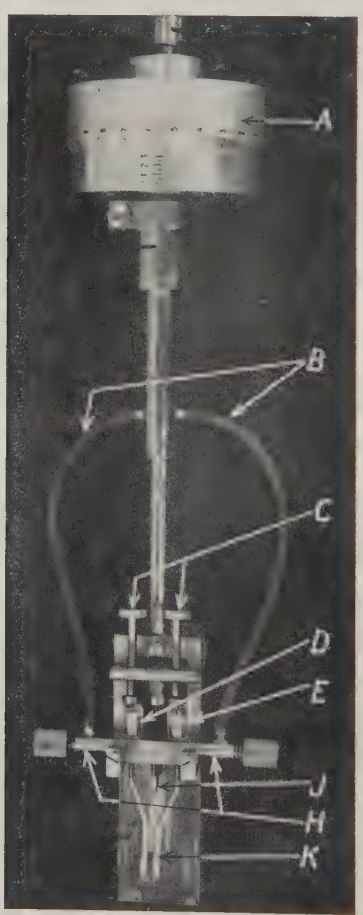


Fig. 2.

TUFT—MICRO-RESPIROMETER WITH AUTOMATIC SETTING AND RECORDING APPARATUS

grid of the final valve, which is arranged normally to have a standing bias of -15 V., under these conditions it is cut off and the motor relay therefore opens; the relay operates at a grid voltage of -3, i.e. for a rectified signal voltage of 12 V. At maximum gain the system should thus be operative for a light input in the order of 10^{-4} lumens, in practice about 10^{-3} is available at the photcell.

The circuit has been thoroughly decoupled, particular care being taken to isolate the relay operating surges from the common h.t. supply. Battery operation is used throughout for both the h.t. and l.t.

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EXPLANATION OF PLATE 11

Fig. 1. The experimental layout showing three micro-respirometers in position. *A*, supporting frame; *B*, micro-respirometers; *C*, lamp housings; *D*, Perspex light ducts; *E*, microscope; *F*, thermostat and heater unit.

Fig. 2. A micro-respirometer, $\times 0{\cdot}9$. *A*, micrometer head; *B*, rubber tubes connecting the vessels with the atmosphere; *C*, clamping screws; *D*, compensating vessel; *E*, reaction vessel; *H*, needle valves; *J*, pipette capillary; *K*, U-tube.

SOME PROPERTIES OF THE PHOTORECEPTORS OF THE BROOK LAMPREY

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(With Ten Text-figures)

This paper describes an investigation of the physiological characteristics of the light receptors situated in the skin of the ammocoete larva of the brook lamprey, *Lampetra planeri* (Bloch). The interest of these organs lies in the fact that they are the only type of simple photoreceptor among the Vertebrates of which we possess any precise information. They may well be considered a kind of functional link between the simpler types of photoreceptor systems, which are found in many Invertebrates, in *Pholas*, *Mya* and *Ciona*, for example, and the fully developed image-forming eye of Vertebrates. We are at present almost completely ignorant of the photosensitive processes of animals at levels of organization below that of the image-forming eye, and an analysis of the properties of these simpler types of receptors offers one of the most promising approaches to an understanding of the common pattern of photoreceptor processes in general.

The eyes of the ammocoete are rudimentary organs buried beneath the skin of the head, and do not appear to act as light receptors. The creature normally remains in its tube in the mud or sand of the stream-bed during the daytime, but may swim freely in the water at night. They were sometimes seen by day swimming in the water, usually under the shade of a bridge or overhanging tree; but when disturbed in the course of collecting operations, most ammocoetes promptly buried themselves in the mud once more.

Few observations have been published on the reactions to light of the lamprey or its ammocoete larva. Parker (1905) found that a swimming reaction was started by shining a beam of light on the tails of quiescent lampreys. Young (1935) demonstrated that the whole skin is more or less sensitive to light, but that the region of greatest sensitivity is in the dorsal fluke of the tail at about its widest point. He showed also that the reaction is a simple photokinesis without directional properties, and that the sensory pathway from the receptors to the central nervous system is by the lateral line nerves and not by the spinal cord. Francis & Horton (1936) published an account of the reactions of ammocoetes to various stimuli, including some experiments on their relative sensitivity to light of different colours. They concluded that the animals are insensitive to light of wave-length longer than 550 mμ, and most sensitive in the blue-green region of the spectrum. Their data, however, were not recorded in a form from which the spectral sensitivity of the reaction can be calculated.

The experiments described below were designed to measure the spectral sensitivity, intensity discrimination, dark adaptation and the effect on the reaction time of varying the intensity and duration of the stimulus; and to present the information in a form which would enable comparison to be made with similar data from other animals.

MATERIAL AND METHODS

All the animals used in the following experiments were ammocoetes of *Lampetra planeri* (Bloch), collected from the River Tyne, East Lothian. They were kept for use as required in the laboratory in large glass aquarium jars filled with river water over a bottom of the fine sandy mud, containing rotting leaves and other debris, from which they were collected. They can be kept in this way for an indefinite period, and require no feeding or other attention.

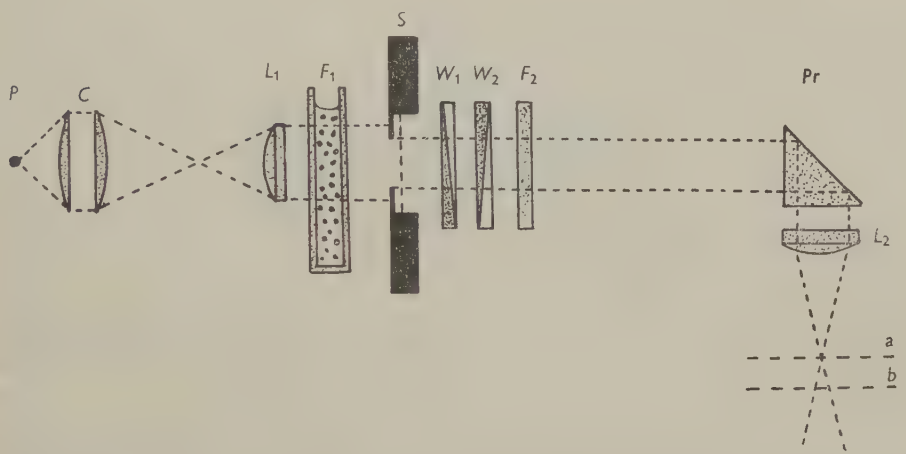


Fig. 1. To show the optical arrangement of the apparatus for light stimulation of the tail of the ammocoete. Key to lettering: *a*, *b*, planes of introduction of ammocoete tail, giving different sizes of test patch; *C*, condenser; F_1, glass cell with optically flat surfaces, containing water; F_2, monochromatic filter or combination of filters; L_1, L_2, achromatic lenses; *P*, Pointolite, 150 c.p.; *Pr*, internally reflecting right-angled prism; *S*, camera shutter with diaphragm and mounting for wedges and filters; W_1, neutral wedge of graded density 0–6; W_2, counter wedge of same density gradient as W_1.

The optical arrangement of the apparatus used for light stimulation is shown diagrammatically in Fig. 1. A 150 c.p. a.c. Pointolite was arranged to project horizontally an accurately paralleled beam about 2 in. in diameter. In the path of the beam were placed a glass cell containing water to absorb heat and ultra-violet radiation, a camera shutter with adjustable diaphragm, neutral wedges and colour filters as required. The light intensity was varied by a neutral wedge of graded density 0–6, which could be slid horizontally along a pair of grooves attached to the mounting of the camera shutter. A counter-wedge of the same density gradient was mounted immediately in front of the first to provide a uniformly illuminated test field. The filter holders were also attached to the same mounting. Immediately above the dish containing the animal the beam was reflected vertically downwards by a 90°

prism with silvered back, and brought to focus by a convergent lens. The size of the test patch was varied simply by raising or lowering the mounting which held the dish containing the ammocoete.

The following general procedure was adopted in all experiments. The ammocoete to be tested was placed in water in a shallow rectangular glass vessel, which was a little longer than the animal and wide enough to permit its natural swimming movements. The depth of water was just sufficient to allow it to maintain its normal posture when at rest. Despite the small volume of water, a few millilitres only, small ammocoetes showed no signs of respiratory distress during experiments which sometimes lasted for several hours. The surface area of the vessel was relatively large, and presumably the animal's periods of activity disturbed the surface of the water sufficiently to maintain the necessary interchange of gases.

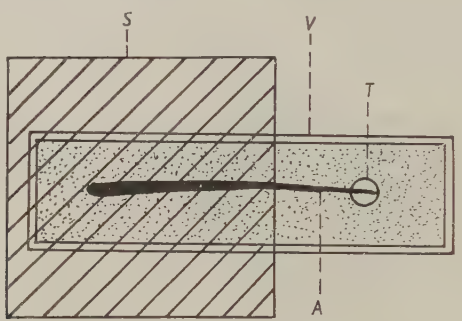


Fig. 2. Surface view of ammocoete in cell mounted for test (scale: approximately life size). Key to lettering: *A*, ammocoete; *S*, screen of black cardboard; *T*, illuminated test patch; *V*, shallow glass vessel containing water.

The test vessel was mounted on a platform attached to a movable stage, which permitted a few centimetres of movement in both horizontal planes. The ammocoetes were not usually disturbed, provided the adjustments of position were carried out slowly and without jerking. For making tests the apparatus was adjusted so that the test patch illuminated the whole of the animal's tail, which lay diametrically across it with the tip on the far circumference, as shown in Fig. 2. This arrangement could be set up simply and repeatedly, so that the same area of skin was stimulated at every test. The size of the test patch was adjusted so that the centre of the field illuminated the dorsal lobe of the tail at its widest point. For an ammocoete of 50–60 mm. length, the test patch was about 5 mm. in diameter. Adjustments of position were made in deep red light, which was obtained by placing an Ilford no. 609 filter in the path of the beam. Nearly all the radiation transmitted by this filter is of wave-lengths longer than 660 mμ, and did not disturb the ammocoete. If the animal settled in a different position after a burst of swimming, the vessel was adjusted by means of the controls of the movable stage until the tail was brought back to its correct position relative to the test patch.

Swimming reactions were observed in dim red light from a 2 V. lamp mounted above the apparatus. This also was fitted with a piece of Ilford no. 609 filter, and its

brightness could be adjusted by means of a rheostat to the minimum required by the observer. A piece of black cardboard was placed over the anterior end of the ammocoete to screen it from stray light, although the head is actually less sensitive than the tail. Before starting a series of experiments the animals were left in darkness for at least 20 min., and were again dark adapted for about 10 min. after each group of measurements.

ANALYSIS OF REACTION TIME

To obtain measurements of dark adaptation, spectral sensitivity and other 'visual' functions in a form suitable for quantitative comparison with similar data from other animals, it is necessary to measure the light energy required to produce a constant physiological effect. When, as in the present case, we are concerned with a photokinetic reaction of the whole animal, we have in practice to measure the intensity of stimulus needed to elicit a constant response. The definition of a constant response is therefore of critical importance, and for this reason it was necessary first to investigate the relation between the intensity and duration of the stimulus in initiating a swimming reaction. It was clear from Young's (1935) experiments that the mechanism of the reaction of the ammocoete is similar to those of *Mya*, *Pholas* and *Ciona*, which were analysed by Hecht (1918, 1921 *a*, *b*, 1928). The time interval between the onset of the stimulus and the start of the swimming reaction is made up of an initial sensitization period, followed by a latent period, during which it is believed that the primary products of the photochemical reaction are engaged in initiating nervous impulses, and during which the illumination need not be continued. At high intensities of stimulus the sensitization period is much shorter than the latent period, but at levels close to the threshold of response it may be considerably longer. In the following experiments the minimum threshold of sensitivity of the dark adapted ammocoete was adopted as the index of a constant response, and the intensity/time relation had therefore to be examined for periods of stimulation up to several seconds duration.

The latent period was estimated by exposing dark adapted ammocoetes to flashes of light of high intensity and short duration, and measuring the time interval between the flash and the start of the swimming reaction. Using flashes of 0·2 and 0·1 sec., the latent period for different animals varied between 1·0 and 1·6 sec., measured to the nearest 0·2 sec. Young's estimate was 1·8 sec., and his minimum sensitization period 0·4–0·6 sec. These discrepancies are not important, however, since the sensitization period depends upon the intensity of light available, while the latent period is affected by a number of environmental factors, such as the temperature, and probably varies a little with the size of the animal also. Young worked principally with large ammocoetes 100–150 mm. in length, while mine were all small ones of about 50 mm.

The intensity/time relation of each ammocoete used for other experiments was measured over a range of 2·5 to 3·0 logarithmic units of illumination, which gave reaction times up to nearly 10 sec. at the lowest intensities. Five measurements of reaction time were made at each level of illumination, and the average calculated.

All the animals yielded similar intensity/time curves, though the actual reaction times showed some variation. Typical curves obtained from two ammocoetes are shown in Fig. 3. These show clearly that as the intensity of illumination was increased, the reaction time decreased to a limiting value, which in most cases was about 1·2 sec. At the other end of the range, the reaction times became more variable as the intensity of the stimulating light approached the animal's absolute threshold of sensitivity.

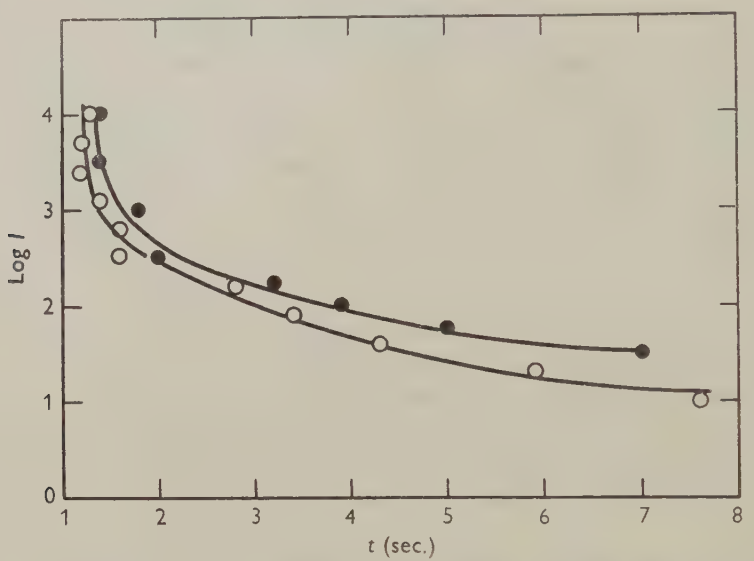


Fig. 3. The relation between reaction time and intensity of light stimulus for two ammocoetes. Each point represents the mean of five measurements.

Inspection of the data showed that they do not conform simply over the whole of this intensity range with the Bunsen-Roscoe Law, which states that to produce a given photochemical reaction a certain amount of light energy is required, and the same result is obtained whether the intensity is high and the time of exposure short, or vice versa. Few, if any, reactions of animals obey this law in its simplest form,

$$E = kIt, \tag{1}$$

where E represents the amount of photochemical effect, I the intensity and t the duration of the stimulus, and k is a constant. The reaction of the ammocoete, however, bears a close resemblance to that of *Mya*, which was investigated by Hecht (1921 *b*). In *Mya*, the photochemical effect is proportional to the product of the logarithm of the intensity and the duration of the stimulus, according to the equation

$$E = kt \log I \tag{2}$$

But in *Mya*, as also in the ammocoete, the total reaction time, r, is made up of a sensitization period, t, folowed by a latent period, p; from which it can be shown that

$$r = k\frac{E}{\log I} + p. \tag{3}$$

Equation (3) is of a straight line type, from which it follows that if the reaction is adequately described by equation (2), a graph of the total reaction time plotted against the reciprocal of the logarithm of the intensity should yield a straight line, cutting the time axis at p units above zero. Hecht found this to be the case for *Mya*. In Fig. 4 the data for the same two ammocoetes as in Fig. 3 are presented in this

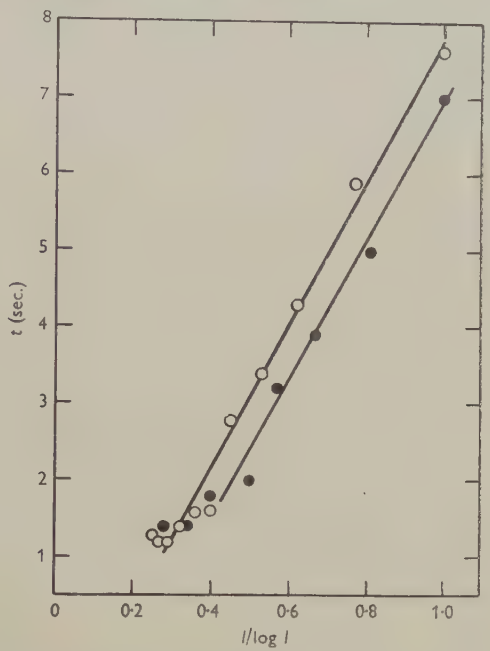


Fig. 4. The relation between reaction time and the reciprocal of the intensity of the light stimulus for two ammocoetes.

form. It is clear that the relation is adequately expressed by a straight line for reaction times longer than about 2 sec., and for practical purposes we may conclude that the intensity/time relation of the ammocoete conforms to the Bunsen-Roscoe Law in the form expressed by equation (2) for relatively long reaction times and low intensities of illumination.

It follows from this analysis that the stimulus required to elicit a constant response can be defined so as to take into account variations in the reaction time in successive measurements. The criterion adopted was the intensity of illumination required to initiate a swimming reaction in $3{\cdot}0 \pm 1{\cdot}0$ sec., expressed in terms of a reaction time of 3·0 sec. In practice the procedure was as follows:

The camera shutter was opened and a stop-watch started simultaneously, the latter being stopped as soon as a reaction was observed. If there was no response, the shutter was closed at 5 sec., the wedge controlling the intensity was moved to a new position and the ammocoete left for about 2 min. in darkness before repeating the stimulus. Reactions which occurred less than 2·0 or more than 4·0 sec. from the onset of the stimulus were disregarded, while those which took place between these limits were adjusted to the

intensity value corresponding with a reaction time of 3·0 sec. Reaction times were measured to the nearest 0·2 sec., and the correction was made empirically from the intensity/time relation curve for each ammocoete.

DARK ADAPTATION

The increase in sensitivity of the photoreceptors in darkness, following a preliminary period of light adaptation, was measured for three ammocoetes. The animals were light adapted by illuminating the whole test cell from above with an unshaded 15 or 100 W. lamp mounted at a distance of 12 in. They were restless, and frequently swam continuously during the period of light adaptation, but usually settled down within a minute or two in darkness. Measurements of the threshold of sensitivity were begun as soon as the ammocoete ceased swimming, and were continued at intervals as near to 2 min. as possible until dark adaptation was judged to be complete. To make a measurement, the wedge controlling the light intensity was set initially at a level of illumination estimated to be below the threshold, and the shutter was opened for 5 sec. If no response was observed, the level of illumination was increased by about 0·15 log unit and the test repeated after an interval of about half a minute. This procedure was repeated until a swimming response was obtained, when the position of the wedge and the time elapsed since the beginning of dark adaptation were recorded. After each response the animal was left in darkness for at least a minute before starting the next measurement.

This procedure is cumbersome compared with the methods developed in recent years for measuring the dark adaptation of the human eye, since relatively few readings could be completed during the period when the sensitivity of the tail was increasing most rapidly. Preliminary trials, however, enabled the observer to estimate the threshold fairly accurately, and thus effect considerable economy in the number of subthreshold stimuli required before obtaining a response.

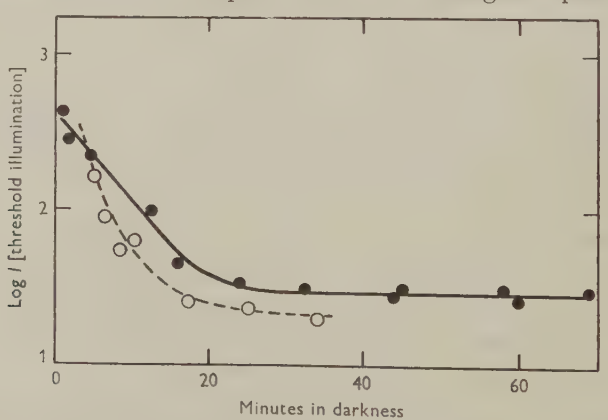


Fig. 5. Dark adaptation of a single ammocoete following 3 min. (open circles, dotted line) and 12 min. (filled circles, continuous line) of preliminary light adaptation by a 100 W. lamp at 12 in. distance.

Two typical dark adaptation curves from the same ammocoete, shown in Fig. 5, serve to illustrate the principal features of the process. In all cases adaptation

followed a simple course, and was complete in 20–30 min. It was more rapid, and the final steady state attained more quickly following short periods and low intensities of preliminary light adaptation than after longer periods and higher intensities. The increase in sensitivity was small compared with the range of dark adaptation of most Vertebrate eyes, being only about 1·0–1·5 log units of intensity.

Although the measurements throughout this work were recorded in arbitrary units of illumination, the brightness of the test patch was measured with a Sei Visual Photometer. The values so obtained for the final dark-adapted threshold ranged from 0·25 to 0·95 millilambert, i.e. approximately 0·25–1 equiv. f.c.

INTENSITY DISCRIMINATION

Intensity discrimination was measured in the following manner. The ammocoete was first adapted to a certain level of illumination for 15 min. The illumination was then increased in successive steps of 0·15 log unit for 5 sec. each until a response was obtained. The same source of light was used both for adapting and stimulating, the shutter being kept open throughout the experiment. The test field was larger than that used in other experiments, being adjusted to illuminate the whole tail end of the animal, whether it was swimming or lying still. As in the dark adaptation experiments, the ammocoetes were restless at higher levels of adapting illumination, but usually remained quiet in dim light. Readings were obtained for two ammocoetes in steps of 0·3 log unit over a range of 3·6 log units. Fig. 6 illustrates the

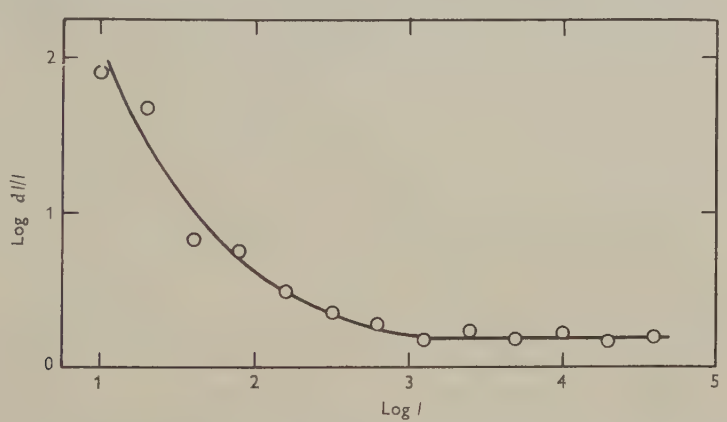


Fig. 6. Intensity discrimination of a single ammocoete at different levels of pre-adapting illumination. Each point represents the mean of five measurements.

result of one such experiment, in which the differential threshold, or increase in stimulus required to elicit a response, log dI/I, is plotted against the level of adapting illumination, log I. At high intensities the relation conformed closely to the Weber-Fechner Law, log dI/I = a constant, but at lower levels the increment of illumination required to obtain a response increased progressively. There was no sign of a similar increase in log dI/I at the highest intensities.

SPECTRAL SENSITIVITY

Spectral sensitivity curves were obtained by measuring the threshold stimulus required to elicit a response from dark adapted ammocoetes with monochromatic light of different wave-lengths. Spectral bands were isolated by means of combinations of filters, based on the Ilford 'monochromatic' series. The optical density and range of spectral transmission of the combinations used were measured with a Hilger Constant Deviation Wave-length Spectrophotometer, and their characteristics are listed in Table 1. The relative spectral sensitivity of the ammocoetes was calculated

Table 1. *Characteristics of the filter combinations used to isolate spectral bands*

Filter (Ilford no.)	Central wave-length (mμ)	Relative transmission
601	440	44
602	470	33
603	497	30
604	519	24
604+605	536	1
605	550	22
606	577	30
607+802	600	46
608+802	652	33

from the average of several observations at each wave-length. The averaged value was then corrected for the relative transmission of the filter combination, the energy distribution and quantum effectiveness of the light source. The energy emission of the 150 c.p. Pointolite used in these experiments approximated closely to that of a gas filled tungsten filament lamp at 2600–2700° K.

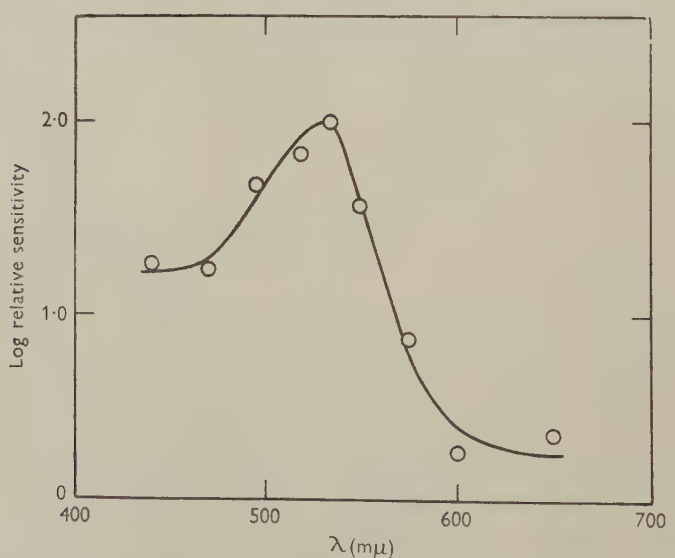


Fig. 7. Spectral sensitivity of the ammocoete. Composite curve constructed from the combined observations on three ammocoetes. Corrected for energy distribution of light source and expressed as equal quantum intensity spectrum.

The spectral sensitivity curve shown in Fig. 7 was constructed from the combined observations on three ammocoetes. and has been adjusted to equal 2·0 on a logarithmic scale at the maximum. The same data are expressed in Fig. 8 as a percentage of the sensitivity at the maximum. The maximum lies in the green, close to 530 mμ, and the sensitivity falls away steeply towards both longer and shorter wave-lengths, so that at 600 mμ it is only about 2 % of the maximum. There is, however, some indication that the sensitivity rises again in the extreme violet, at wave-lengths shorter than 450 mμ.

DISCUSSION

The measurements described in this paper provide information on which to base deductions concerning the nature and mode of action of the photoreceptors. The shape of the curves obtained for dark adaptation and intensity discrimination suggest that the system contains a single type of receptor and photopigment only, since both functions vary in a continuous manner with respect to time and intensity of adapting illumination respectively. Neither of them shows any sign of a break, or sudden change in slope, such as is commonly found in similar experiments on eyes which contain both rods and cones in the retina, and distinct scotopic and photopic visual mechanisms. The range of dark adaptation of the ammocoete is strikingly small, amounting to an increase in sensitivity after light adaptation of some 10 to 30 times. For comparison, human dark adaptation can, under suitable experimental conditions, show about a million-fold increase in sensitivity, and with relatively simple apparatus can be measured over a range of 10,000 times (4 log units). The final stable threshold of sensitivity of the fully dark adapted ammocoete is also surprisingly high. Francis & Horton (1936) estimated it at about 3 equiv. f.c. The values in my experiments are rather lower, ranging from about 0·25 to 1 equiv. f.c., which is still some 100,000 times less sensitive than the dark adapted threshold of medium-sized retinal fields in the parafoveal region of the human eye. These findings suggest that the photoreceptor system of the ammocoete is not concerned with detecting or discriminating between low levels of illumination, but simply with activating the animal in the presence of bright light.

The general conclusion which may be drawn from the measurements of dark adaptation is that there are relatively few receptor cells compared with the number of retinal elements of an image-forming eye, and that the individual receptors probably lack the capacity to accumulate large amounts of the photosensitive substance. In these respects the physiological findings agree with the morphological, since there are certainly no obvious concentrations of cells in the tail of the ammocoete of any type likely to be photoreceptors, nor has any photosensitive pigment yet been identified or extracted from them.

The spectral sensitivity curve is of special interest. The maximum sensitivity of the ammocoete at about 530 mμ falls between the maxima of scotopic and photopic vision of the human and most Vertebrate eyes. The photopigment concerned cannot be ordinary visual purple (rhodopsin), the absorption maximum of which is close to 500 mμ, nor does it correspond with any known photopic visibility curve,

since all such curves possess maxima at wave-lengths longer than 530 mμ and are sensitive to radiation between 600 and 700 mμ in the red end of the spectrum. The position of the maximum at 530 mμ does, however, strongly suggest a photopigment of the visual violet (porphyropsin) type, in which vitamin A_2 replaces vitamin A_1 in the chromophore. The absorption maximum of porphyropsin lies between 520 and 530 mμ, at 522 ± 2 mμ according to Wald (1939), which is reasonably close to the maximum sensitivity of the ammocoete. Porphyropsin visual systems are characteristic of fresh-water fish, and although direct evidence is not available for the brook lamprey or its ammocoete larva, one would expect them to contain vitamin A_2. Wald (1942) found that the eyes of the anadromous sea lamprey, *Lampetra marinus*, contain predominantly porphyropsin and vitamin A_2, though the liver and other

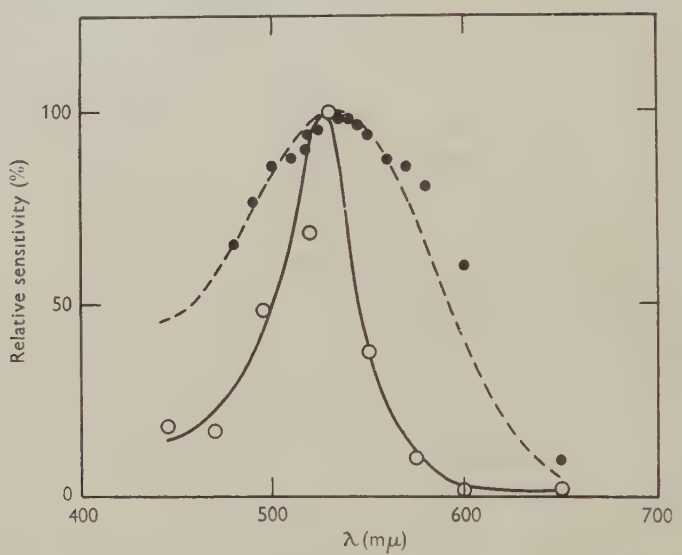


Fig. 8. Spectral sensitivity of the ammocoete (open circles, continuous line) compared with the scotopic sensitivity of the tench (broken line) and with the percentage absorption of tench porphyropsin (filled circles). Scotopic sensitivity of the tench redrawn from Granit (1941). Percentage absorption of tench porphyropsin calculated from the data of Bayliss *et al.* (1936). Equal quantum intensity spectrum.

tissues contain principally vitamin A_1. Spectral sensitivity curves, however, have been obtained for the eyes of some species of fresh-water fish, which possess the porphyropsin visual system. The scotopic sensitivity of the carp, *Cyprinus* species, and the tench, *Tinca vulgaris*, were measured by Granit (1941) by means of the micro-electrode technique on the excised opened eye. The curve for the tench corresponds closely with the absorption spectrum of porphyropsin, prepared from the same species by Bayliss, Lythgoe & Tansley (1936), and resembles the spectral sensitivity of the ammocoete in certain important respects. The two curves are compared in Fig. 8, which shows also (filled circles), data by Bayliss *et al.* for tench porphyropsin plotted as a percentage of the maximum absorption. The maximum sensitivity of both the tench and ammocoete is close to 530 mμ, and both curves

tend to flatten about 450 mμ or even to rise again in the violet. The principal difference between them is that the curve for the ammocoete falls away more steeply on either side of the maximum, so that its spectral range of sensitivity appears to be more restricted than that of the tench, or than the absorption spectrum of porphyropsin.

This difficulty may be resolved by taking into account the effect of differences in the concentration or in the thickness of the absorbing layer of the photosensitive substance. Hecht, Shlaer & Pirenne (1942) pointed out that the shape and width of the percentage absorption curve of a substance varies with its concentration, and the spectral sensitivity must represent the percentage absorption curve of a particular concentration of the photopigment. This fact is obscured when the absorption spectra are presented in the form of extinction or optical density curves, as is the usual practice. They computed percentage absorption curves for various concentrations of visual purple, which show clearly that when the maxima are adjusted to the same value (100 %) irrespective of the actual fraction of the light absorbed, the width of the curves increases as the concentration is increased. Similar data are not available for porphyropsin, but the hypothesis that this is the photopigment concerned in the light reaction of the ammocoete can be tested empirically by adjusting the percentage absorption curve of a solution of porphyropsin for various concentrations or thicknesses until the best fit is obtained. The most comprehensive series of absorption spectra for porphyropsin are probably those of Wald (1939), who investigated five species of North American fresh-water fish. The curves all agree closely with one another for wave-lengths longer than about 500 mμ, and the maxima all lie at 522 ± 2 mμ. They diverge to some extent at shorter wave-lengths, all five showing a rise in absorption in the violet, which is probably due in part to impurities. The rise in the violet is greatest in the pickerel, *Esox reticulatus*, and least in the calico bass, *Pomoxis sparoides*, and the white perch, *Morone americana*. The last two probably most nearly represent the absorption spectrum of pure porphyropsin, and I have therefore selected the curve for the calico bass for comparison with the spectral sensitivity of the ammocoete. The data were first converted from extinction to a percentage absorption basis (Table 2), and the resulting curve is shown as the interrupted line in Fig. 9. From this curve a family of percentage absorption curves for different concentrations or thicknesses of porphyropsin can be derived in the same way as for visual purple; since each layer of equal thickness absorbs an equal fraction of the light, precentage absorption increases by the square as the thickness is doubled, and so on. Fig. 9 illustrates such a transformation, in which the spectral sensitivity of the ammocoete is compared with the percentage absorption of a porphyropsin solution of thickness one-sixth that of Wald's curve for the calico bass. It is clear that the correspondence between the two is very close over the whole spectral range.

This analysis demonstrates that the properties of porphyropsin provide an adequate basis for the light reaction of the ammocoete. No special significance can be attached to the fact that the animal's sensitivity curve fits the sixth power of the percentage absorption spectrum, but we may conclude that the conditions in the photoreceptors

of the tail are such that the concentration and effective thickness of the absorbing layer combine to yield an action spectrum which corresponds with a percentage absorption curve for porphyropsin, whose maximum absorption is several times lower than that of preparations from the eyes of fresh-water fish.

Table 2. *Spectral absorption of porphyropsin of the calico bass,* (Pomoxis sparoides), *calculated from the data of Wald* (1939)

Wave-length (mμ)	Extinction log I_0/I	Percentage absorption (A)	Percentage absorption of thickness A/6
400	0·62	76·1	19·4
430	0·55	71·8	13·7
440	0·55	71·8	13·7
460	0·62	76·1	19·4
480	0·78	83·4	33·6
500	0·93	88·2	47·0
520	1·00	100·0	100·0
540	0·95	88·8	49·2
560	0·80	84·1	35·4
580	0·55	71·8	13·7
600	0·30	48·8	1·3
620	0·15	29·1	0·6
640	0·08	14·5	0·1
660	0·02	3·8	0·03

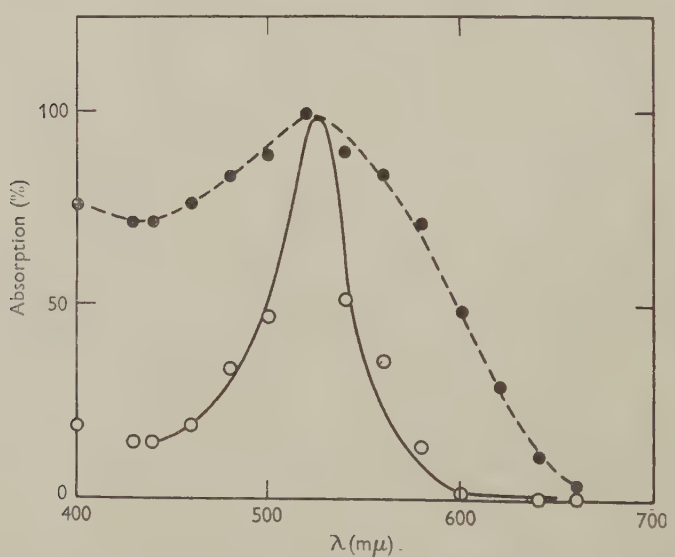


Fig. 9. Spectral sensitivity of the ammocoete (continuous line) compared with the percentage absorption of porphyropsin from the calico bass (broken line, filled circles) and with the sixth power of the same solution (open circles). Percentage absorption of porphyropsin of the calico bass calculated from the data of Wald (1939). Equal quantum intensity spectrum.

It is instructive to pursue this line of inquiry by comparing the spectral sensitivity of the ammocoete with the curves for various Invertebrates. We find that the simple light reactions of the Molluscs *Mya arenaria* and *Pholas dactylus*, investigated by

Hecht (1921 *a*, 1928), also exhibit sharply defined maxima and relatively narrow spectral limits. These reactions resemble that of the ammocoete in being mediated by diffusely scattered photoreceptors in the skin, and not by image-forming eyes. That of *Pholas* in particular closely resembles the ammocoete, with the maximum close to 530 mμ and with a tendency to rise again in the violet (Fig. 10). The curves

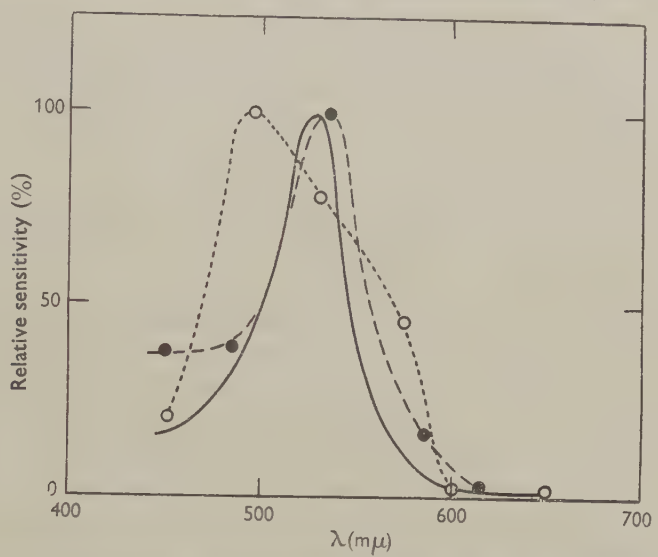


Fig. 10. Spectral sensitivity of the ammocoete compared with that of *Pholas* (broken line, filled circles) and *Mya* (dotted line, open circles). Data for *Pholas* and *Mya* redrawn from Hecht (1921, 1928). Equal quantum intensity spectrum.

are indeed so similar that one is tempted to suggest porphyropsin as the photopigment in *Pholas* also, at a slightly higher concentration than in the ammocoete. This would be surprising, since the bulk of the evidence available indicates that porphyropsin and vitamin A_2 are associated exclusively with fresh water or anadromous Vertebrates, and the matter clearly merits further investigation. In *Mya*, on the other hand, the position of the maximum close to 500 mμ suggests a system based on rhodopsin (visual purple). Although the sensitivity curve was constructed from measurements at six wave-lengths only, it corresponds not unreasonably with the percentage absorption curve for a dilute solution of visual purple.

Sensitivity measurements of the eyes of various Invertebrates show that the latter tend to resemble Vertebrate eyes in possessing broad spectral bands, which do not fall away so steeply on either side of the maximum. Thus the spectral sensitivity of the eye of *Limulus* is very similar to the human scotopic visibility curve, (Graham & Hartline, 1935), and that of *Dytiscus* is a broad band with the maximum about 540 mμ, (Granit, 1947). This suggests that broad spectral sensitivities are in general characteristic of image-forming eyes, whether Vertebrate or Invertebrate, which contain large numbers of retinal elements and relatively high concentrations of photosensitive pigments; while narrow bands with sharply peaked maxima are found in the simpler systems, in which the receptor cells are few and scattered, and the concentra-

tion of photosensitive pigment low. So far as it is possible to analyse them at present, the properties of these simple photoreceptor systems appear to be compatible with the known photosensitive pigments of image-forming eyes, and it seems unnecessary to invent special substances to account for their physiological differences.

SUMMARY

1. A method is described for measuring the principal characteristics of the light reaction of the ammocoete, and data are presented on the relation between the intensity and duration of the stimulus, dark adaptation, intensity discrimination and spectral sensitivity.

2. The intensity/time relation approximates to the Bunsen-Roscoe Law at low intensities of illumination. Use is made of this fact to define a criterion for a constant response to a given stimulus, for measuring the other physiological properties of the system.

3. Dark adaptation and intensity discrimination at different levels of adapting illumination both yield simple curves, which suggest that the system contains a single photosensitive pigment. The range of dark adaptation following a period of light adaptation is from 10 to 30 times. The threshold of excitation of a dark adapted ammocoete is from 0·25 to 0·95 millilambert.

4. The spectral sensitivity possesses a sharply peaked maximum about 530 mμ. Reasons are presented for suggesting porphyropsin (visual violet) as the photosensitive pigment concerned.

I wish to thank Prof. James Ritchie and Dr T. D. M. Roberts for much helpful advice and criticism in the preparation of this paper.

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THE INFLUENCE OF PHOTOPERIOD AND TEMPERATURE ON THE INDUCTION OF DIAPAUSE IN *DIATARAXIA OLERACEA* L. (LEPIDOPTERA)

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(With Five Text-figures)

INTRODUCTION

Many insects, like other organisms, pass through a resting period during some stage in the life cycle in order to survive adverse conditions. In insects this resting period may occur during either the egg, larval, pupal or adult stages, and it is noteworthy that even nearly related species may show little or no similarity either in the stage at which dormancy occurs or the physiological state of the dormant period. Thus in the lepidopterous family Agrotidae the dormant period may occur during the larval stage of *Agrotis segetum* Schiff., and during the adult stage of *Plusia gamma* L. These species become quiescent during conditions of low temperature in winter, but on the advent of warm conditions they continue to develop without delay. However, other Agrotidae may show diapause—a physiological stage of arrested development which is not terminated immediately merely by increasing the temperature. Thus, *Euxoa nigracans* diapauses as an egg, whereas *Diataraxia oleracea* L. diapauses as a pupa. In view of such diversity in mode of surviving adverse conditions it is perhaps not unexpected that the factors inducing diapause should differ according to the insect species. A review of work on this subject has been given by Wigglesworth (1939) and Dickson (1949), and thus it is sufficient to say here that in many insects behaviour with regard to diapause is inherited, while in others diapause may be partly or wholly induced by adverse environmental conditions such as low temperatures, unsuitable nutrition and low moisture content in the food.

For several insects it has been shown already that the length of the photoperiod may influence diapause. Marcovitch (1924) showed that artificial reduction of day length to 7·5 hr. caused four species of aphis to produce males and oviparous females which laid diapause eggs, while under long-day conditions only viviparous parthenogenetic females were produced. Similar results were reported by Davidson (1929) and Wadley (1931).

Dickson & Sanders (1945) showed that diapause in *Grapholitha molesta* Busck., was affected by temperature and photoperiod during the larval stage, and the results of further detailed studies on this species are reported by Dickson (1949), who showed that high or low temperatures during the larval feeding period prevented occurrence of diapause. At medium temperatures larvae reared in the absence of light did not enter diapause, but as the photoperiod was increased to more than 3 hr.

the percentage diapause increased and reached 100% at about 11–12 hr. of light per 24 hr. At about 13 hr. the percentage of larvae entering diapause dropped suddenly to practically zero and remained at this level with further increases of light. *Carpocapsa pomonella* showed a similar response to photoperiod.

Preliminary observations on the influence of photoperiod on diapause were reported by Way, Hopkins & Smith (1949), who showed that at 24° C. a photoperiod greater than 16 hr. per 24 hr. during the larval stage was sufficient to prevent diapause in pupae of *Diataraxia oleracea* L. The photoperiod also influenced diapause in *Pieris brassicae* and *Mamestra brassicae*.

The present work was carried out to determine the effects of both light and temperature on diapause in *Diataraxia oleracea*. Particular attention has been paid to a determination of the period during larval development when the photoperiod is operative as a factor influencing diapause.

MATERIAL AND METHODS

D. oleracea L., the tomato moth, is a species belonging to the family Agrotidae. Under natural conditions the adults are active during June and July, and there is one and sometimes a partial second generation each year. In artificially heated glasshouses, the adults begin to emerge from overwintering pupae in January and February and there are generally three generations per year (Lloyd, 1920; Speyer & Parr, 1947).

All experiments were carried out in constant temperature rooms or cabinets. Larvae were reared in light-proof cages, each of which was artificially illuminated from above by a 60 W. tungsten filament bulb. A large glass trough full of water prevented radiant heat from the bulb causing increase in temperature in the cage. The light intensity to which the larvae were subjected was recorded as 25 f.c. (foot candles). This represents an approximate mean, largely because it was not possible to maintain larvae on the food plant at a fixed distance from the light source. Mention will be made in the text whenever different light intensities were used.

The larvae were reared on cut foliage of various brassica varieties—generally cabbage or brussels sprouts. A dish of dry peat was provided for pupation and each day the newly formed pupae were removed and kept at 24° C. until emergence of the adults. The exact length of the pupal period of each individual was recorded.

EXPERIMENTAL

(*a*) *Mode of diapause of* Diataraxia oleracea

In a valuable study of the habits of *D. oleracea*, Lloyd (1920) found that individuals varied greatly in the length of the pupal stage which lasted from 16 to 300 days. Two categories were distinguished: the short-period pupae from which adults emerged 16–50 days after pupation, and the long-period pupae from which adults emerged after 90–300 days. Although in these studies temperature conditions varied widely, Lloyd was able to show that low temperatures were not required to break diapause in the long-period pupae.

The present investigations have been carried out at constant temperatures. At 24° C. (±0·5° C.) adults emerged from short period pupae 14–30 days after pupation, 86% emerging between 17 and 21 days. Emergence of adults from long-period pupae began about 50 days after pupation and continued for a further 100 days. This distinction between the long-period or diapause pupae and the short-period or non-diapause pupae was always well defined except in experiments at 24° C. where the larvae were reared in continuous darkness. Under such conditions some adults emerged from the pupae during the intervening period 30–50 days after pupation (see Fig. 3).

(*b*) *The influence of photoperiod at* 24° C.

Lloyd (1920) found that under glasshouse conditions where the mean monthly temperature varied from 21·5 to 22·9° C. (range 11–34° C.), pupae obtained between May and July were primarily non-diapause while in April and September all were of the diapause type. Table 1 shows the percentage pupae entering diapause during each month between April and September (Lloyd, 1920) compared with the approximate mean day length per month determined from the average number of hours between sunrise and sunset (1950, for London Area).

Table 1. *Comparison of percentage of pupae of* Diataraxia oleracea *entering diapause (from Lloyd, 1920) with 'day lengths' for the months April to September*

	April	May	June	July	August	September
Average hours—sunrise to sunset	14·0	15·5	16·5	16·0	14·5	13·0
Percentage diapause pupae	100	1·2	0·0	2·3	66·4	100

Clearly the significance of day length as expressed above is limited, since the actual day length depends on the sensitivity of the larva to light of low intensities at the beginning and end of the day. Moreover, irrespective of the time of the year, 'day length' would be reduced when the sky is overcast or when the larvae are shaded by foliage of the food plant.

It would appear that if day length during the larval stage is important, a photoperiod of at least 15·5 hr. per 24 hr. is required to prevent onset of diapause, and it is noteworthy that experiments carried out at 24° C. have shown that using artificial light a photoperiod of 16 hr. or more during the larval stage of *D. oleracea* largely prevented diapause in the resulting pupae (Way *et al.* 1949).

The critical photoperiod was determined by rearing batches of about fifty *D. oleracea* larvae at a constant temperature of 24° C. under photoperiods varying from 0 to 24 hr. per 24 hr. using artificial illumination of 25 f.c. Fig. 1 shows the percentage pupae entering diapause in each treatment, and it can be seen that a photoperiod of 15 hr. is critical (cf. Table 1). The sharp drop in percentage of pupae entering diapause which occurs as the photoperiod during the larval stage is increased to more than 14 hr. occurs also in *Grapholitha molesta* Busck. in which (at 24° C.) a photoperiod of 13–14 hr. per 24 hr. is critical (Dickson, 1949).

Fig. 1 shows that, although photoperiods of 4–14 hr. during the larval stage induce 100% diapause, photoperiods of less than 4 hr. may prevent diapause in up to 20% of the pupae.

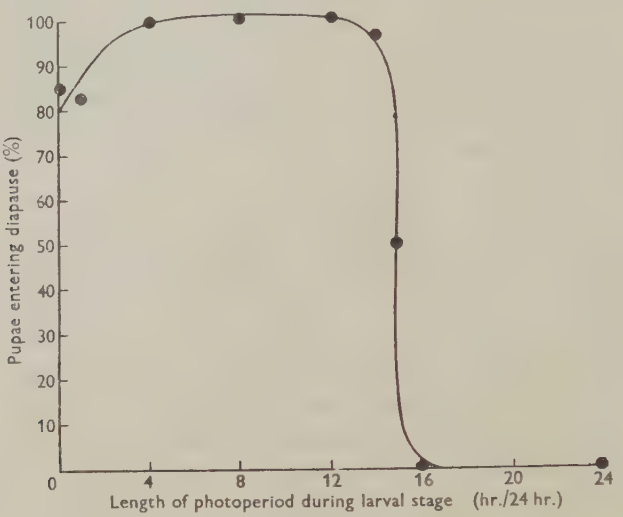


Fig. 1. The influence of photoperiod during the larval stage on the percentage pupae of *D. oleracea* entering diapause. Temperature during larval and pupal stages, 24° C.

(*c*) *The influence of temperature on induction of diapause*

Experiments were carried out to determine the effects of rearing larvae at 30 and 34° C. using photoperiods which induce diapause at 24° C. The prepupae* and pupae from the larvae reared at 34° C., and the pupae from all the other treatments were kept under identical conditions at 24° C.

Fig. 2 shows the effect on the length of the pupal stage, of rearing larvae at 24, 30 and 34° C., using a standard photoperiod of 8 hr. light (25 f.c.) per 24 hr. It can be seen that pupae resulting from larvae reared at 24 and 30° C. were all of the diapause type, the length of the pupal period varying from 49 to 147 days. However, when the larvae were reared at 34° C., 49% of the resulting pupae were non-diapause (pupal period 16–21 days), while the other 51% were of the diapause type (pupal period 54–135 days).

A further experiment was carried out in which larvae were reared under the same range of temperatures but in darkness (except for a few minutes per day). Fig. 3 shows that at 24° C., 15% of pupae were non-diapause and 85% were of the diapause or intermediate type. However, at 30 and 34° C. all pupae were non-diapause.

Thus, although conditions of illumination are such that at 24° C. they induce diapause, diapause may be prevented if the temperature during the larval stage is raised to 30 or 34° C.

Further experiments were carried out to determine the effect of temperatures

* At 34° C. mortality at ecdysis from the prepupa to the pupa is practically 100%, although the larvae develop satisfactorily at this temperature (Way, Smith & Hopkins, 1950).

lower than 24° C., and Table 2 shows the percentages of diapause pupae resulting from batches of larvae reared at 12, 15, and 18° C. using a photoperiod (16 hr. per 24 hr.) which at 24° C. prevents diapause. Only small numbers of insects were used, and furthermore larval development was not satisfactory at 12 and 15° C., but it is

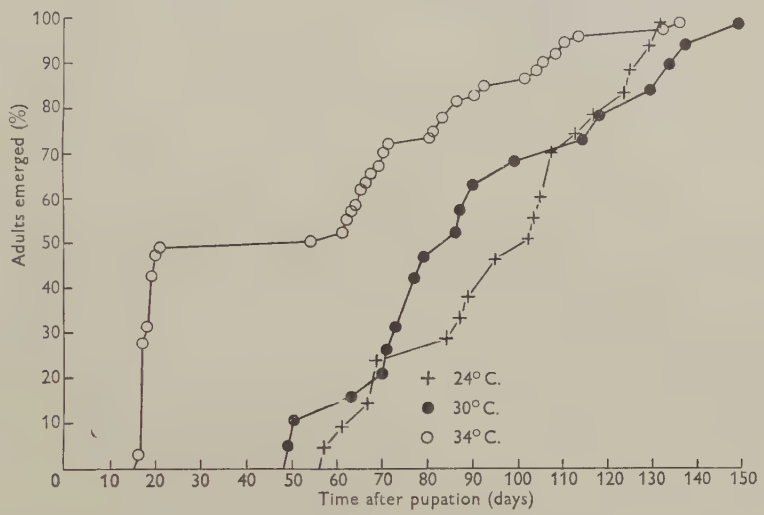


Fig. 2. The influence of different temperatures during the larval stage on the duration of the pupal stage of *D. oleracea*. Photoperiod during larval stage, 8 hr. per 24 hr. Temperature during pupal stage, 24° C.

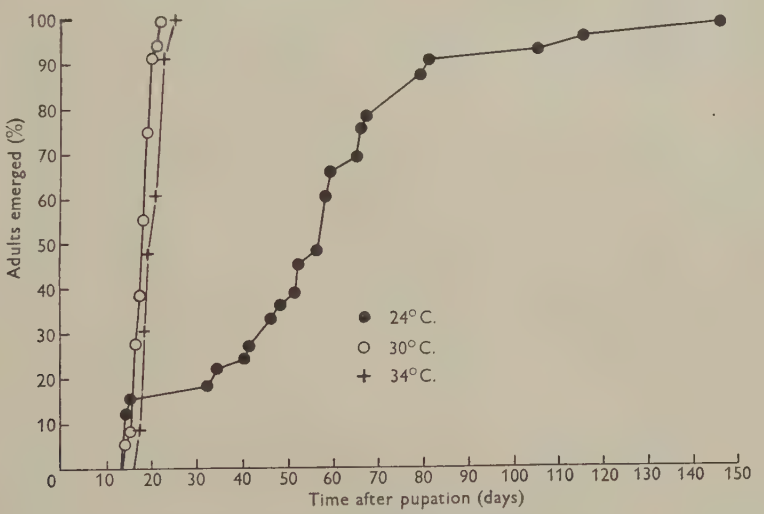


Fig. 3. The influence of different temperatures during the larval stage on the duration of the pupal stage of *D. oleracea*. Larvae reared in darkness. Temperature during pupal stage, 24° C.

clear that low temperatures tend to induce diapause even under long photoperiods. This behaviour would appear to be advantageous to the insect, and yet it is remarkable that diapause in the *G. molesta* larva (at any rate in California, U.S.A.) is prevented by a low temperature (Dickson, 1949).

Table 2. *The percentages of diapause pupae resulting from larvae of* Diataraxia oleracea *reared at low temperatures and with a photoperiod of* 16 *hr. per* 24 *hr.*

Temperature during larval stage (° C.)	12	15	18	24
No. of pupae	13	13	9	13
Pupae in diapause (%)	92*	92	44	0

* Two of these were of intermediate type and emerged after 38 and 39 days.

(*d*) *The influence of light intensity on diapause*

It has been shown that when larvae of *Diataraxia oleracea* were subjected to illumination of 25 f.c. for a photoperiod of 16 hr. per 24 hr. at 24° C. the resulting pupae were non-diapause. However, when the photoperiod was reduced to 12 hr., diapause was induced. To determine whether light intensity and light energy influenced diapause, batches of 20–30 larvae were reared at 24° C. using photoperiods of 12 and 16 hr. per 24 hr. and at three light intensities—220 f.c. (1000 W. tungsten lamp), 25 f.c. (60 W. tungsten lamp), and 1 f.c. (15 W. tungsten lamp)—a large jacket of running water did not entirely absorb radiant heat from the 1000 W. lamp since a thermometer with blackened bulb placed in the rearing cage showed a temperature of 25–26° C. compared with the external temperature of 24° C.

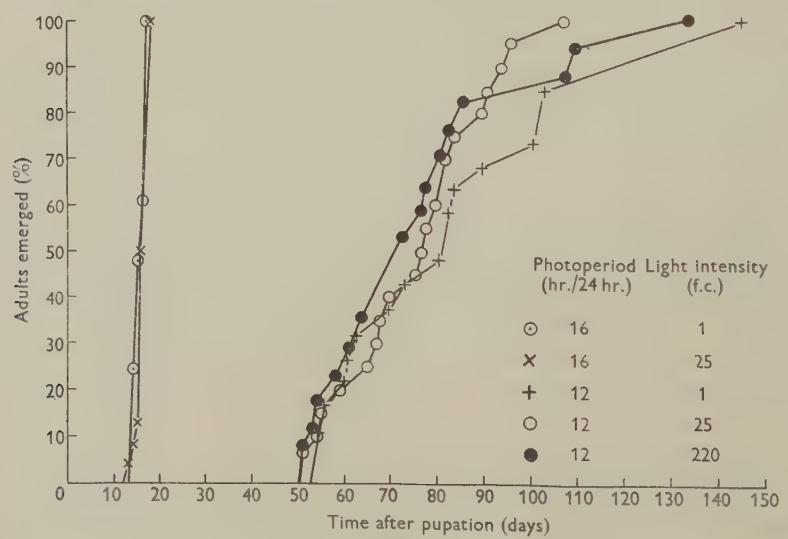


Fig. 4. The influence of light intensity and length of photoperiod during the larval stage on the duration of the pupal stage of *D. oleracea*. Temperature during larval and pupal stages, 24° C.

The data in Fig. 4 and Table 3 show that at a photoperiod of 12 hr. all pupae were of the diapause type (pupal period 50–147 days) irrespective of the light intensity (1–220 f.c.). However, at a photoperiod of 16 hr. an intensity of 1 f.c. was sufficient to prevent diapause.

Table 3. *Comparison of the influences of light energy and light duration during the larval stage on diapause in pupae of* Diataraxia oleracea

Light intensity (f.c.)	1	1	25	25	220
Ratios of total light energy $\left(\frac{\text{f.c.} \times \text{photoperiod}}{12}\right)$	1	1·3	25	33	220
Length of photoperiod (hr. per 24 hr.)	12	16	12	16	12
Pupae entering diapause (%)	100	0	100	0	100

The results suggest that light intensity and light energy during the larval stage have no influence on diapause which is influenced solely by light duration, though it is clear that there must be a minimum intensity (less than 1 f.c.) below which the *D. oleracea* larva is insensitive to light.

(*e*) *Period of larval stage during which the photoperiod is operative*

Detailed experiments were carried out in which larvae of *D. oleracea* reared at 24° C. were transferred from a diapause-preventing photoperiod (16 hr.) to a diapause-inducing photoperiod (8 hr.) and vice versa at various stages in larval development. It was found that larvae transferred from an 8 hr. to a 16 hr. photoperiod at the beginning of the moulting sleep at the end of the penultimate instar became non-diapause pupae. Conversely, larvae transferred from a 16 hr. to an 8 hr. photoperiod at the same stage of development became diapause pupae. Thus, the photoperiod is not operative as a factor influencing diapause until after the beginning of the moulting sleep.

At 24° C. the moulting sleep prior to ecdysis to the last instar lasts about 2 days, and the last instar about 9–10 days of which the first 5–6 days are spent in feeding and the last 4 days in formation of a cocoon and as a prepupa. Batches of about fifty larvae reared at 24° C. were transferred from a photoperiod of 16 hr. (diapause-preventing) to one of 8 hr. (diapause-inducing): (1) at the end of the penultimate instar at the beginning of the moulting sleep; (2) towards the end of the moulting sleep 0–12 hr. before ecdysis to the last instar; (3) after 1 day in the last instar; (4) after 3 days in the last instar; (5) after 5 days in the last instar.

Table 4 shows that diapause is not prevented if larvae of *D. oleracea* are reared under a diapause-preventing photoperiod only during the period prior to the moulting sleep into the last instar. However, a diapause-preventing photoperiod during the first 36–48 hr. of the moulting sleep is sufficient to prevent diapause in 81 % of the pupae.

Further experiments were carried out in which larvae reared under a diapause-inducing photoperiod (8 hr.) were transferred to a diapause-preventing photoperiod (16 hr.) at various stages in their development: (1) at the end of the penultimate instar; (2) after 3 days in the last instar; (3) after 5 days in the last instar. Fig. 5 shows that pupae from treatment 1 were all of the non-diapause type, from treatment 2

were 50% diapause and from treatment 3 were 100% diapause. Thus, the diapause-preventing photoperiod is not operative after the 5th day of the last instar and only partially effective after the 3rd day of the last instar.

Table 4. *The percentage of diapause pupae resulting from larvae of* Diataraxia oleracea *reared under a diapause-preventing photoperiod and transferred to a diapause-inducing photoperiod during various stages of the last instar of* Diataraxia oleracea. *Temperature, 24° C. Light intensity, 25 f.c.*

Duration of photoperiodic conditions (hr. per 24 hr.)		No. of pupae	Percentage pupae entering diapause
16 (diapause-preventing)	8 (diapause-inducing)		
To beginning of moulting sleep at end of penultimate instar	For moulting sleep and throughout last instar	21	100
To within 12 hr. before ecdysis to last instar	For 12 hr. at end of moulting sleep and throughout last instar	26	19
To end of 1st day of last instar	For last 9 days of last instar	30	0
To end of 3rd day of last instar	For last 7 days of last instar	24	4
To end of 5th day of last instar	For last 5 days of last instar	25	0

To summarize: at 24° C. the photoperiod is operative as a factor influencing diapause only during the period between the beginning of the moulting sleep at the end of the penultimate instar and the 3rd–5th day of the last instar.

It is of interest that when larvae were subjected to the diapause-preventing photoperiod throughout the whole of the last instar the mean length of the pupal

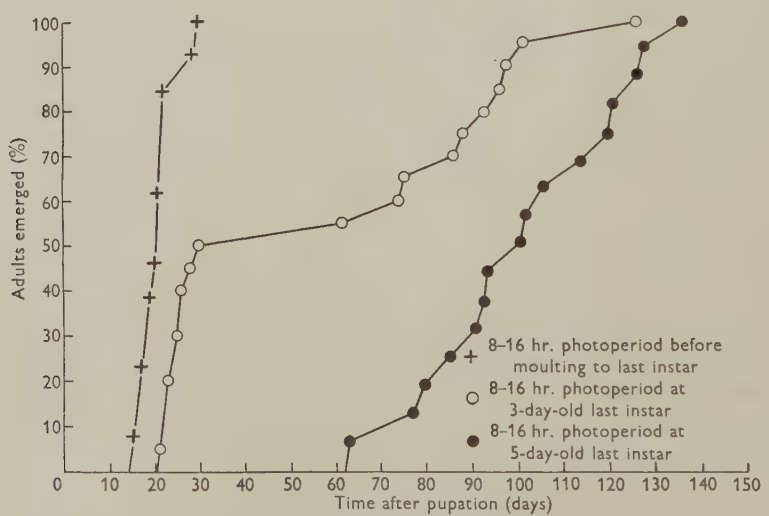


Fig. 5. The effect on the length of the pupal stage of transferring the larvae of *D. oleracea* from a diapause-inducing photoperiod (8 hr. per 24 hr.) to a diapause-preventing photoperiod (16 hr. per 24 hr.) at various times during the last larval instar. Temperature during larval and pupal stages, 24° C.

stage was 21 days, whereas the mean length of the pupal stage of non-diapause pupae resulting from larvae reared in a diapause-inducing photoperiod for the first 3 days and a diapause-preventing photoperiod for the remainder of the last instar was 25 days. Thus a delay of 5 days before subjecting the last instar to a diapause-preventing photoperiod caused an increase of 4 days in the length of the pupal period (see Fig. 5). This suggests that, under diapause-preventing conditions, the initial stages of adult differentiation begin during the moulting sleep prior to ecdysis to the last larval instar. On this basis adult differentiation at 24° C. takes about 32 days, of which the first 12 days occur during the larval period and the last 20 days during the pupal period.

(*f*) *The influence of the food plant*

To determine whether the effect of the photoperiod might be a secondary one due to the influence on the larva of photoperiodically controlled substances in the food plant, larvae were reared under a diapause-preventing photoperiod (16 hr. at 24° C.), the food plant being raised under a diapause-inducing photoperiod (8–12 hr.) and changed in the larval rearing cages twice each day in such a way that the larvae never consumed food plant which had been subjected to a photoperiod greater than 12 hr. The resulting pupae were all of the non-diapause type. Similarly, several experiments were carried out in which larvae reared under a diapause-inducing photoperiod were given food plant which had been reared under a diapause-preventing photoperiod. All the resulting pupae entered diapause.

Therefore diapause in *D. oleracea* is not influenced by photoperiodically controlled substances in the food plant. The photoperiod as a factor influencing diapause must act directly on the larva.

DISCUSSION

Bodine (1932) and Salt (1947) have suggested that diapause is induced by formation of a diapause factor or hormone which causes development to be arrested. Diapause continues as long as the factor is present, but the factor may be slowly dissipated over a period of time or rapidly destroyed by exposure to low temperature thus enabling development to proceed.

Dickson (1949) has suggested that in *Grapholitha molesta* a diapause hormone may be produced during the larval stage by a two-phase reaction—a light-induced phase requiring a photoperiod of between 7 and 15 hr. and a darkness-induced phase requiring a dark period of between 11 and 16 hr. However, the present work with *Diataraxia oleracea* has shown that over 80% diapause occurred when the larvae were reared in the absence of light at 24° C. Thus the theory of a light and dark reaction does not appear to be valid. However, on the basis that a diapause hormone is responsible for the arrest of development, it might be said that when the larva of *D. oleracea* is subjected to a photoperiod of less than 15 hr. a hormone is secreted which inhibits growth and induces diapause, while at photoperiods greater than 15 hr. the hormone is not secreted and development proceeds normally.

However, it is not easy to reconcile this theory with the evidence that: (1) a single diapause-preventing photoperiod during the moulting sleep, prior to ecdysis to the last instar of *D. oleracea*, was sufficient in 81 % of the cases to cause development to proceed normally even though the larvae were subjected to a diapause-inducing photoperiod for the rest of the instar; (2) a diapause-inducing photoperiod up to the 3rd day of the last instar did not induce diapause in 50% of pupae providing the larvae were subjected to a diapause-preventing photoperiod for the 4th and 5th days of the last instar. Similarly, in *Grapholitha molesta* (Dickson, 1949) a diapause-inducing photoperiod (presumably causing secretion of the diapause hormone) during the whole of the last 8–13 days of the larval feeding period was incapable of inhibiting growth and inducing diapause even though the larva was subjected to a diapause-preventing photoperiod for only the first 4 days of the larval period. Moreover, a diapause-inducing photoperiod for the first 4 days did not inhibit the effect of a diapause-preventing photoperiod to which the insect was subjected for the last 8–13 days. Now it would seem that if the diapause-inducing photoperiod caused the production of a hormone which inhibited further growth, diapause should have been induced in all the above experiments.

Clearly it is necessary to examine these results with regard to the theory of diapause put forward by Wigglesworth (1934), who showed that arrest of growth due to lack of hormones occurred in *Rhodnius prolixus* and suggested the diapause may result from the absence of hormones necessary to maintain growth rather than to the presence of hormone which inhibits diapause. On the basis of this theory, it may be said that pupae of *Diataraxia oleracea* enter diapause when the hormone which initiates adult differentiation is absent. However, if the last instar larva, reared at 24° C., is subjected to a photoperiod greater than 16 hr. the adult growth hormone is liberated and diapause is prevented. Once production of the hormone has been initiated, the diapause-preventing conditions are no longer required because development does not cease even if the larvae are afterwards subjected to conditions which tend to induce diapause.

The theory that the absence of growth-promoting hormones is responsible for diapause has been strongly supported by Williams (1946), who studied pupal diapause in several lepidoptera and found no evidence that diapause resulted from the presence of a factor inhibiting adult differentiation. Williams showed that the factor or hormone responsible for adult development was secreted by the pupal brain, and by means of transplants he demonstrated that diapause was due to the failure of the brain to supply this factor. The presence of a growth-promoting hormone in lepidoptera was also demonstrated by Kopec (1922), who showed that metamorphosis in *Lymantria dispar* was induced by a hormone produced by the larval brain. If the brain was removed before the hormone had been secreted, development was arrested and a state comparable to that of diapause was induced.

Thus it seems probable that diapause in the pupa of *Diataraxia oleracea* is due to the absence of a hormone responsible for adult differentiation rather than to the presence of an inhibiting hormone. As regards the physiological effect of the photoperiod it is suggested that a photoperiod of 16 or more hr. per 24 hr. prevents

diapause because the prolonged stimulation of the last instar larval brain by light falling on the photoreceptors induces formation of the adult growth hormone. This condition would be analogous to that occurring in certain birds and mammals where several investigators (see Rowan, 1938) have shown that an artificial increase of the photoperiod corresponding to the change from winter to spring conditions caused the eyes to stimulate the anterior pituitary gland by nervous paths (Hill & Parkes, 1933; Le Gros Clark, McKeown & Zuckerman, 1937) to produce a hormone which induced sexual development.

Perhaps constant high temperatures during the larval stage of *D. oleracea* by increasing nervous activity also induce the brain to secrete the growth-promoting hormone. The fact that low temperatures tend to cause diapause even in the presence of long photoperiods supports this theory, since it might be expected that low temperatures would reduce nervous activity to such a level that even prolonged stimulation from the photoreceptors would not activate the brain sufficiently to cause secretion of the growth-promoting hormone.

It is remarkable that if the larvae are reared in darkness, diapause is partly prevented at 24° C. and completely prevented at 30–34° C. This phenomenon is even more marked in *Grapholitha molesta* (Dickson, 1949), where at 24° C. diapause is completely prevented at photoperiods less than about 6 hr. per 24 hr. It is of interest that darkness has a similar effect on sexual activity of certain birds and mammals. Thus, although sexual activity in ferrets (Hill & Parkes, 1934) and house sparrows (Ivanova, 1935) is suppressed by a short photoperiod and induced by a long photoperiod, it is also induced by rearing in darkness (23·5 hr. per 24 hr.) or by covering the eyes with an opaque hood.

SUMMARY

The induction of diapause in the pupa of *Diataraxia oleracea* is influenced by temperature and photoperiod during the larval stage. Low temperatures and short photoperiods tend to induce diapause while high temperatures and long photoperiods tend to prevent diapause.

Diapause is not influenced by light intensity during the larval stage providing the intensity is above a certain minimum.

Diapause is prevented at high temperatures (30–34° C.) if the larvae are reared in darkness.

The photoperiod is operative as a factor influencing diapause only between the beginning of the moulting sleep prior to ecdysis to the last instar and the 3rd–5th day of the last instar. A single diapause-preventing photo-period during the moulting sleep is probably sufficient to prevent diapause.

Diapause in *D. oleracea* is not influenced by photoperiodically controlled substances in the larval food plant.

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PHASES OF ACTIVITY IN THE SEA-ANEMONE, *METRIDIUM SENILE* (L.), AND THEIR RELATION TO EXTERNAL STIMULI

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(With Eleven Text-figures)

We have shown in a previous paper (Batham & Pantin, 1950*b*) that the sea-anemone *Metridium senile* exhibits continual very slow muscular activity. It is most evident in the changes of shape of the column caused by the reciprocal contractions of the circular muscle sheet, and of the longitudinal parietal musculature which runs at the junction of each mesentery with the body wall. This inherent activity varies, so that in the same individual it presents a different pattern on different occasions. The pattern may remain constant for hours on end, and may then change over a comparatively short period. Thus a moderately filled anemone giving fairly regular and substantial contractions may pass into a condition with a larger volume and much less apparent activity, or it may pass into a condition of prolonged and almost complete contraction. These patterns of activity often endure for long periods—hours or even days. It is convenient to refer to such patterns as 'phases', and these phases form the subject of this paper.

METHODS

Our conclusions are based on direct observation, quick-motion cinematography and kymographic records. These methods have been discussed fully in the previous paper (Batham & Pantin, 1950*b*). For kymograph tracings, long light isotonic levers were used. When continuous tracings for days or weeks were required, slow clockwork drums were employed, and the writing levers were arranged to swing at right-angles to the surfaces of the smoked drums. For all the tracings figured in this paper, the recording thread ran vertically from the sphincter region of the anemone to the lever. Hence the records represent chiefly the vertical activity of the body wall (Fig. 1*a*). All tracings have been photographed and arranged in the figures so that they read from left to right, as indicated by arrows, and so that contractions are registered downwards. To improve reproduction, the long fine tracings of the records were broadened by being thrown slightly out of focus and photographed on panchromatic process plates through a red filter. Throughout the work described in this paper the specimens of *Metridium* used were of medium size, the diameters of their pedal disks lying between 2 and 4·5 cm. They were collected from low spring tide level at Mill Bay

and Salcombe, Devon, a few days or weeks before they were used. Very prolonged recordings were made under controlled conditions described in a subsequent section.



Fig. 1. Appearance of *Metridium* during various phases. *a*, specimen showing position of hook or thread for recordings illustrated in all other figures in this paper. *b*, specimen ingesting food. Contraction of radial muscles of perfect mesenteries opens actinopharynx. *c*, typical appearance ¼–½ hr. after food ingestion. *d*, *e*, swaying movements 6 hr. after food-extract stimulus. *f*, *g*, *h*, successive stages in anti-peristaltic constriction, leading to defaecation and (*i*) 'shrivelling'. *j*, subsequent re-filling, showing extreme expansion 4 hr. after 'shrivelling'. *k*, specimen showing excretory ring of mucus and uric acid (*u*.). *l*, state of extreme contraction, typical of unfed specimen exposed to sunlight.

FOOD AS A STIMULUS TO PHASE CHANGE

The phase of activity of an animal may sometimes change, as we shall see, without any evident external stimulus. In other cases the new phase may be clearly engendered by a particular stimulus to which its character bears a significant relation. This happens in the response of the animal to the presence of food. Fig. 2 records an instance of this. At first the animal was in a somewhat contracted state showing fairly regular alternations of parietal contraction, and re-extension by contraction of the circular muscle. At the point marked 'Fed', about 1 g. of tissue of *Mytilus* was placed on the disk. The mouth opened by contraction of the radial muscles of the perfect mesenteries (Batham & Pantin, 1950*a*), and the food was completely swallowed within 10 min. (Fig. 1*b*). The immediate mechanism of appropriation of food by anemones and its ingestion has been discussed by Parker (1919) and others (Pantin & Pantin, 1943). We need not add to these accounts.

The sequence of events following ingestion in the experiment recorded in Fig. 2 was typical. The first effect of ingestion was relaxation of the sphincter and the flowering out of the capitulum, the disk and the tentacles. Thirteen minutes later

contractions of the circular muscle appeared in the column (Fig. 1 *c*). Quick-motion cinematograph films of this stage show active, though very slow peristalsis. By pressure on the coelenteric fluid, this peristalsis causes elongation of the column. Elongation in the case illustrated in Fig. 2 was almost complete in an hour, during which time the volume of the animal had increased as well as its height.

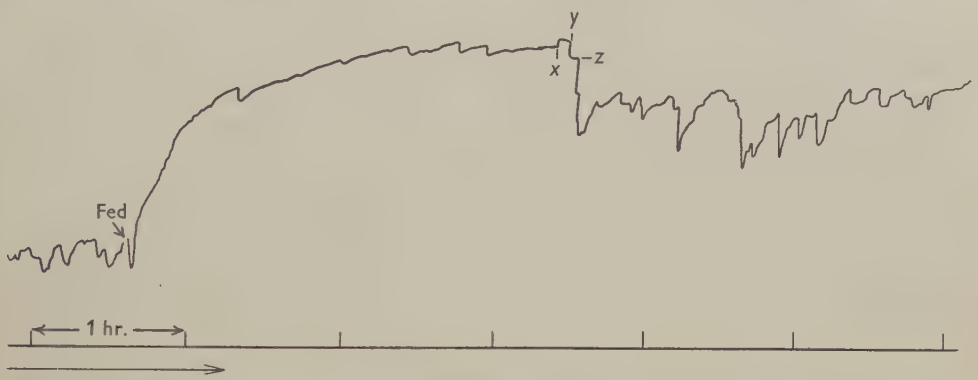


Fig. 2. Kymograph record of response of *Metridium* to feeding. At point marked 'Fed' about 1 g. of *Mytilus* tissue was placed on oral disk. *X*, *Y*, *Z*, local parietal contractions causing swaying movements.

This change of height and volume resulted from a change of the animal's phase of activity. Examination of such cases as this shows that during elongation, the number of alternating contractile events in the antagonistic muscle system of the column which take place in a given time does not undergo great change. But there is marked change in their relative extent. The parietal contractions are now much smaller, and may do no more than check extension, whilst the circular contractions are more extensive and more powerful.

In Fig. 2 the animal remained in this phase of activity for nearly 3 hr., when a new phase supervened. At first unilateral (*X*, *Y*, *Z*) bending movements took place. In more marked instances of this, a tall *Metridium* very slowly sweeps or sways over and round to one side after another (Fig. 1 *d*, *e*). These movements are sometimes just fast enough to be appreciable to the eye. They seem to be caused in part by unilateral contractions of the parietal and the parieto-basilar muscles. Cinematograph records also make it clear that they are in part due to loss of circular tone, as a result of which the coelenteric turgor is unable to support the animal and it collapses under its own weight. When in this state each wave of circular contraction causes a re-erection of the column followed by a fresh collapse.

In the experiment of Fig. 2, large parietal contractions began to occur after the initial swaying, so that the elongated animal was now very active. These contractions resulted in some shortening and widening of the shape.

Three to six hours after a meal, a *Metridium* has increased greatly in volume and settled down to a phase of only slight apparent activity (cine film). This seeming lack of activity, however, may be partly of mechanical origin. When the animal is

greatly distended, muscular contraction of the body wall causes a rise in pressure against which the muscles cannot easily shorten (Batham & Pantin, 1950*a*).

In due course, further changes of phase take place in connexion with defaecation. The ingested food is sometimes extruded only partially digested. In one series of observations on twenty-six animals this occurred in ten of them between 24 and 40 hr. after ingestion. More usually faecal pellets surrounded by thick mucus are extruded within 48 hr. Immediately after defaecation 'shrivelling' takes place (Batham & Pantin, 1950*a*). This is a rapid and generalized contraction of the whole musculature with loss of coelenteric water so that the anemone becomes a shrivelled caricature of its normal state (Fig. 1*i*). Shrivelling may be complete in less than 3 min., after which the animal refills itself; a process taking about 2 hr. in instances observed. Subsequent acts of defaecation occur during the fortnight following a meal; the extruded faeces becoming smaller and darker. A yellow excretory ring of mucus and uric acid (Fox & Pantin, 1941) develops rapidly round the column 1½ to 2 days after a meal. Similar rings develop in starved animals but much more slowly (Fig. 1*k*).

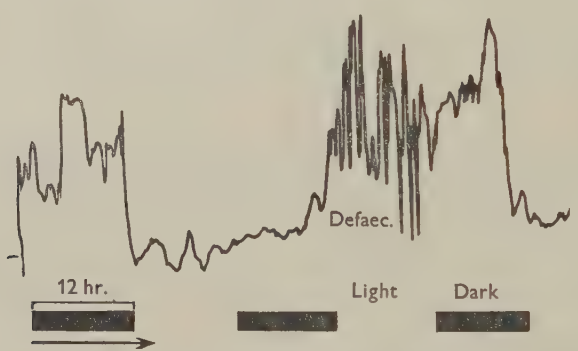


Fig. 3. 2½-day record of activity of fed specimens, showing extensive movements during ejection of food remains (Defaec.).

Defaecation occupies a phase of great activity. Fig. 3 shows the extensive contractions and re-extensions of the body wall which lead up to the process. These are accompanied by great activity of the circular muscle. Deep constrictions may remain localized or travel up or down the column. About half a dozen antiperistaltic waves, each occupying several minutes, may be succeeded by some peristaltic waves. Then further antiperistaltic activity may be shown. A succession of these events leads to defaecation and the sudden loss of water by generalized contraction ('shrivelling'), which is followed by re-inflation (Fig. 1*f–j*).

We see from these observations that ingestion of food is followed by a sequence of phases. There is expansion of the disk, often elongation of the column, sometimes swaying of the column, followed by inflation with some shortening, defaecation, shrivelling and a return to normal. Each of these phases has its own pattern of activity and most may last an hour or more.

This sequence is set in motion by a stimulus: the receipt of food. But the relation between stimulus and response is not that of a simple reflex, like the response of the retractor muscles following strong stimulation; it may involve very different neuromuscular machinery. To begin with, the response is not a simple contraction, but a prolonged and irregular pattern or sequence of patterns of activity. Secondly, these phases or patterns of activity are not simply built up from specific responses to a specific sequence of stimuli. We may contrast them with the response sequence involved in the capture of food by the tentacles (Pantin & Pantin, 1943). In that case, contact of a piece of solid food with a tentacle produces a direct local response. This in turn leads to a combined chemical and mechanical stimulation of other nearby tentacles which transfer the food over the disk towards the mouth. This engenders new local stimuli of the disk and finally of the mouth, which result in ingestion. Each event of the sequence in this case is conditioned by the appropriate local chemical and mechanical stimuli. If the relation of the mechanical and chemical components of the stimulus is artificially changed, the orderly character of the response is upset. If the stimulus (food object) is removed, the response sequence is brought to a premature end (Pantin & Pantin, 1943).

In contrast with this, while the phases of activity we are now considering may be initiated by specific stimuli, they can on occasion be initiated by stimuli of other kinds. Indeed they may appear in the absence of any apparent external stimulus. Shrivelling may be initiated by other stimuli than the presence of food in the last stages of digestion; strong and prolonged stimuli such as repeated intense illumination may initiate it. Swaying, shrivelling and other phasic activities may appear in unstimulated animals, or to stimuli not previously effective. Thus, in Fig. 11 a starved *Metridium* contracting and expanding to daily illumination, in a manner to be described later, abruptly passed into the shrivelled state 1 hr. after the beginning of illumination on the third recorded day.

The patterns of these phasic activities are in some way implicit in the 'excitor-effector' machinery of the animal. They do not depend on a chain of specific external stimuli. Even when an external stimulus initiates a phase, it merely releases a pattern of activity which the animal is quite capable of executing in its absence. The stimulus, as it were, merely pulls a 'hair-trigger'.

Notwithstanding this limited connexion between the external stimulus and the pattern of phasic activity, the phases take place in an orderly sequence, and, as quick-motion cinematography of the food reaction makes clear, the sequence of phases is often evidently purposive and functionally significant. What ensures that each phase is followed by the next in the series remains to be determined. We can only point out that individual phases of the food-response series can on other occasions appear by themselves or in a different sequence: as in the alternating phases of expansion and retraction in relation to light and dark to be discussed below.

This relation of stimulus to the phase initiated is made clear by examining the effect, not of solid food, but of food solutions. It has long been recognized that the presence of dissolved substances from food could cause expansion of the disk in

sea-anemones (Pollock, 1883; Parker, 1919). This effect is frequently, though by no means always, to be observed.

In the following experiments the stimulating solution was obtained from soft parts of *Mytilus edulis*, or of *Helix aspersa*, or the epithelium of *Loligo*. These were ground with sand and with sea water and the resulting fluid filtered through cloth. The sensitivity of different *Metridium* to such solutions varied greatly, but the addition of sufficient extract to raise the concentration of dry organic matter in the medium to about 1 part in 10^6 was generally sufficient.

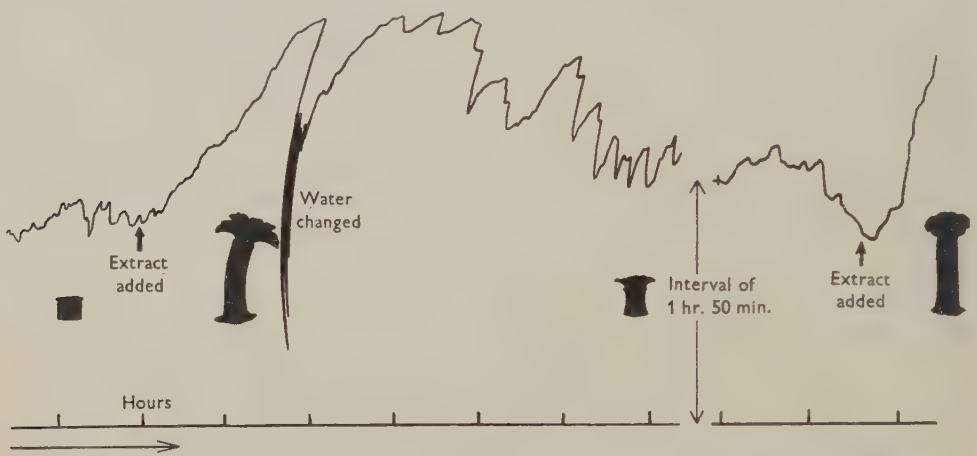


Fig. 4. Record showing effects of food extract. Anemone soon passes to elongated phase. Compare with Fig. 1. For explanation see text.

Fig. 4 shows the effect of *Mytilus* extract, at about 1 in 10^6 dry weight in the medium, on a specimen of *Metridium*. The animal was at first in clean sea water and had assumed a phase of moderate contraction and activity. Within 5 min. of addition of the extract the disk and tentacles began to expand. The tentacles showed some twitching movements. During the next 2 hr. the column of the anemone greatly elongated. This took place in just the same way as in animals fed on solid food. The frequency of contractile activity remained approximately unchanged, but the waves of circular muscular contraction increased in intensity whilst the extent of the reciprocal parietal contractions was greatly diminished.

Two hours after the addition of the food extract the water was completely changed three times. The state of expansion was in no way diminished, and after an hour or so a phase of strong parietal contractions began to develop, just as occurs at the corresponding time after a meal of solid food. And, as in that case, elongation was followed by swaying movements. Swaying is often seen in such experiments, and it is noteworthy that whereas it seems to have little functional significance when engendered by a meal of solid food, it appears strikingly purposive as a response to dissolved food material. By sweeping the ground with its tentacles the anemone by such movements increases the chance of 'finding' the prospective meal the dissolved food might signify.

The first part of the record ends $4\frac{1}{2}$ hr. after the removal of the food extract. By this time the anemone ceased to be elongated, though now moderately expanded. The record begins again about 2 hr. later, and at the point indicated *Mytilus* extract was once more added to the medium to a concentration of 1 in 10^6. There now resulted an even more rapid elongation of the column than on the first occasion. The experiment was then discontinued.

There are some important features to note in this. In the first place, the sequence of phases almost precisely resembles that of the first few hours after the acceptance of solid food. The succession of phases and their nature does not therefore depend upon the stimulus of a solid food object in the actinopharynx or coelenteron.

Not only can the succession of phase changes be caused by a filtered food extract as well as by solid food, but it will be seen from Fig. 4 that the change of phase continues for hours after the removal of the food extract from the external medium. The ability of a stimulus to cause a change of phase which endures long after its termination is seen in Fig. 5 in a case of another kind. The animal in this experiment was moderately open and decidedly active, as the frequent large parietal contractions signify. During the period marked 'Shocks', condenser shocks were administered to the column at a frequency of six a minute for 20 min. This caused an increased state of parietal contraction which slowly passed into extension. For several hours after the stimulus, the pattern of activity remained decidedly different from what it was before stimulation. Parietal contractions were much less frequent.

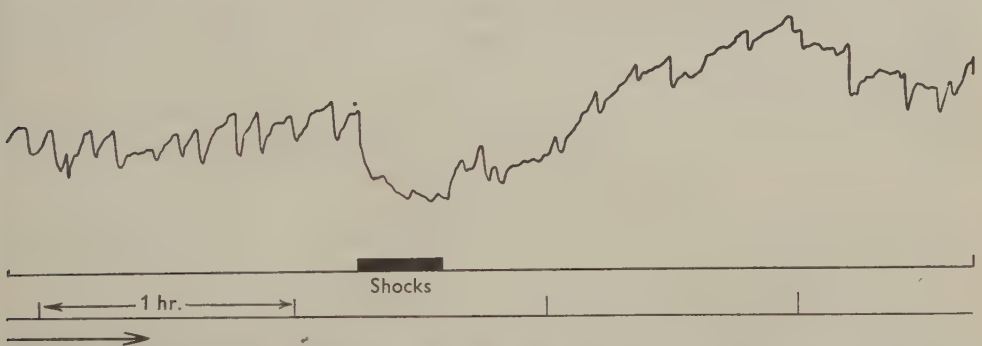


Fig. 5. Kymograph record showing effect of prolonged electric stimulation on phasic activity of column. Series of condenser discharge shocks given at 10 sec. frequency for 20 min.; strength a little above threshold. Note reduced frequency of parietal contractions.

The various phases of activity are thus initiated rather than maintained by the stimulus. Whether a stimulus will initiate a phase, however, varies according to the state of the animal. The complete sequence of phases in response to food substances only takes place in some individuals and at certain times. These differences may in part depend on the temporary shape of the anemone. There is some evidence that suggests that it is primarily the tentacles that receive the stimulus leading to phasic expansion as a response to food substances. Anemones that are completely contracted down (Fig. 1 *l*) show the feeding response much less frequently and more slowly than those in which the disk is exposed. For instance, in one experiment

twenty-three previously starved *Metridium* were exposed to food extract whilst under prolonged observation in a large aquarium. Of these twenty-three, sixteen had their sphincters contracted, thereby covering the tentacles more or less completely at the time when the extract was added. During the next 6 hr. the other seven anemones whose tentacles had been exposed each showed a greater or lesser degree of expansion. Of the sixteen closed specimens, however, four opened very slightly and the other twelve all remained completely closed. In other tests it was found that the injection of food extract through the body wall into the coelenteron was less effective in producing phasic expansion than its addition to the external water bathing the surface of the tentacles.

But the threshold of phase changes to food extracts does not simply depend on the degree of exposure of the tentacles. It is true that, as in the above experiment, individuals whose tentacles are covered by the contracted marginal sphincter usually fail to respond to food extract, while those animals whose tentacles are exposed all give some response. But in animals with exposed tentacles and of the same apparent shape there are great differences in the extent of expansion of the disk and even greater differences in the incidence of other phases of the food sequence. Whilst all may give the expansion of the disk and tentacles, not all give the subsequent elongation of the column. Others again may omit other phases of the sequence, such as the swaying of the column. The threshold for the various phases may vary greatly.

The threshold for a reflex response may vary owing to sensory adaptation. Repeated application of a stimulus may thereby temporarily reduce its effectiveness. This can be seen in the tentacular responses to food (Parker, 1919; Pantin & Pantin, 1943). But the changes of threshold of phasic activity are not solely to be ascribed to this factor. When the animal is in a suitable state, a phase may be initiated repeatedly. We have seen in Fig. 4 that the elongation reaction could be induced with ease twice in succession at an interval of 10 hr. The reaction was again strongly evoked in the same anemone 5 days later. Only after a meal and previous prolonged exposure to food extract (on the 6th day) did a repetition of the experiment elicit expansion of the disk alone, and fail to cause elongation of the column. In contrast with this there were many other cases where elongation of the column was never induced, even though there had been no recent previous exposure to food extract. Such variations of threshold cannot simply be attributed to sensory adaptation. There must be, in addition, some other, internal, factor which governs the ease with which a phase is initiated, and which varies. We must suppose the existence of some such factor when we find the phases of elongation and swaying occurring spontaneously in apparently unstimulated animals. Similarly shrivelling, a phase normally following defaecation, has been several times observed in animals maintained in clean sea water under constant conditions of light and temperature.

We thus come to consider these phases as patterns of activity potentially present in the animal independently of specific external stimuli. The stimuli merely release them. The threshold for their release varies, and may become so low that the phase occurs spontaneously. Moreover, the same phase may be initiated by more than one kind of stimulus.

One essential factor which governs the threshold of initiation of a phase depends upon what we can only at present call the 'state' of the animal. Starved animals are certainly in a different physiological state from well-fed ones, and on the whole starvation progressively lowers the threshold for initiation of the phases associated with feeding, and vice versa. How it comes about that the neuromuscular system can assume different physiological patterns remains to be seen. But we may note some degree of analogy to the changes of pattern in visceral activity in the Vertebrata which follow changes in endocrine balance.

In concluding this section we would say that in our experience *Metridium* will on occasions readily take large pieces of solid food. That it will not always do so is, we believe, satisfactorily accounted for by the variation of its state from time to time. It is not alone amongst carnivores in only being 'on the feed' at intervals. Its behaviour on receiving solid food both when witnessed by the eye and by cinematography gives no grounds for the supposition that ingestion of large food masses is an 'abnormal' process. It may well be that it commonly ingests planktonic organisms but we see no reason to consider it purely a specialized planktonic feeder (cf. Elmhirst, 1925).

LOCOMOTOR PHASES

A phasic activity of a different kind from that which we have just discussed is locomotion. As McClendon (1906) and Parker (1917) state, this takes place through exceedingly slow waves of contraction passing across the foot from behind forwards in the direction of locomotion. This is vividly shown by accelerated cine-photography of walking specimens. Parker finds that the direction of locomotion bears no relation to the siphonoglyph axis of the animal. He also remarks upon the independence between this activity of the foot and the presence of the rest of the animal; animals with the oral disk removed walked as readily as entire ones.

We shall discuss the mechanism of locomotion in a later paper based on the analysis of cinematograph records. Here we shall confine ourselves to consideration of the conditions under which locomotion occurs. In an aquarium, the position of the foot of *Metridium* may remain unchanged for weeks. In the field, the relative positions of members of an asexual clone of one colour suggests that they remain on one site for even longer periods. As Gosse (1860) remarks, some older naturalists actually supposed that the animals were permanently fixed to the substratum. In such cases, sections show the foot to be attached to the substratum by a layer of hardened protein material. Nevertheless, *Metridium* kept in aquaria not infrequently show intermittent locomotor phases (Flatteley & Walton, 1922).

Our own observations suggest that locomotion may be preceded by a condition of pedal instability. Though remaining on the same general site, the foot then shows irregular extensions and retractions in various directions, so that its outline progressively changes over a period of hours. If locomotion supervenes, this localized activity becomes co-ordinated and directed.

Though locomotion shows co-ordination and direction, it may begin under conditions of constant temperature and light intensity and the absence of any evident

external stimulus. Thus a *Metridium* was placed without food in a dimly lit aquarium 8 days after it had been fed. It attached itself and remained stationary until the night of the 13th day, during which it walked. It walked again on the 34th and 35th days. On the 36th day it shrivelled and recovered. On the 37th day it again walked but thereafter remained stationary till the 46th day.

Occasionally locomotion in *Metridium* seems directly initiated in response to a prolonged adverse stimulus. It was often observed that an anemone subjected to repeated electrical or mechanical stimulation or to prolonged very powerful illumination or to the sewing-in of a recording thread would begin walking during the following night. Similarly, a freshly collected animal whose foot has pieces of the substratum still adhering to it often walks for some days until the pedal disk is cleared of debris.

Locomotion initiated by a stimulus may at times bear no directional relation to the

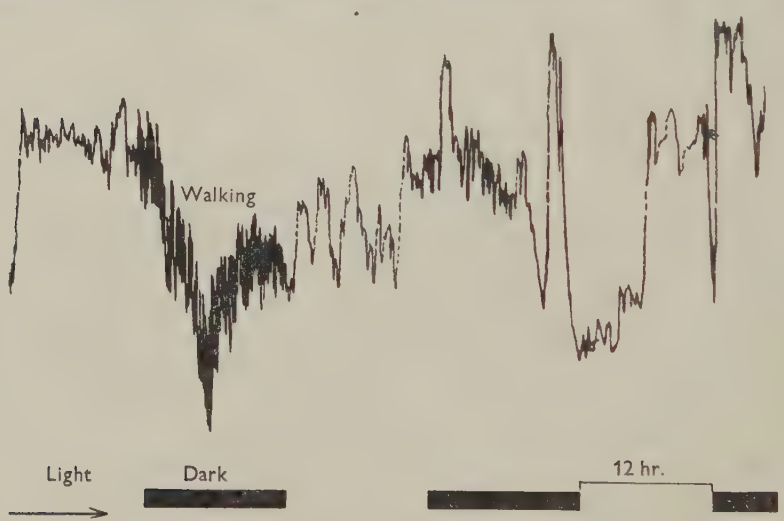


Fig. 6. Kymograph record of *Metridium* submitted to 12 hr. periods of light and dark. Note marked general activity during locomotion.

stimulus, but it is commonly away from it. In certain cases, stimuli which had nothing to do with initiating locomotion may come to control its direction. Thus there is a frequent tendency for locomotory animals that reach the sides of an aquarium to travel upwards vertically till they reach the surface of the water, as though gravity can in some way act as a directing force. Possibly the slight extra tension on the uppermost muscles is here the directing stimulus.

The threshold for the initiation of locomotion is influenced by some general factors. A large meal is often followed by locomotion. Thus eight anemones from one clone were starved for a week. They were then placed in separate bowls of sea water in a cellar under constant conditions. Four were at this juncture given half a *Mytilus* and the rest left unfed. All were observed daily at 9 a.m. and 9 p.m. for 3 weeks except upon 3 days. During these 12 hr. intervals the four fed animals

walked on two, three, five and six occasions respectively. Of the four unfed ones, one walked twice and the rest remained stationary.

Stimuli may not only initiate a phase; they may inhibit it. Apart from the tendency for a strong local stimulus by light to initiate locomotion, general illumination tends to inhibit this activity. It was repeatedly observed that locomotion, when it occurred, almost always did so at night. Thus a starved anemone in an aquarium exposed to daylight began to move on 11 August 1946. It remained stationary during the daytime, but travelled 11·0, 11·5 and 6·5 cm. on three successive nights, by which time it had reached the surface-level of the water. After that no further locomotion occurred during the next 5 days and nights.

The locomotory phase is an activity pattern of considerable complexity. Notwithstanding Parker's observations of isolated pedal disks, cinematograph and other records show that the muscular waves across the pedal disk are accompanied by co-ordinated constrictions running down the column and extensive vertical activity. The latter is shown in Fig. 6, a tracing of the vertical activity of an anemone exposed to alternating 12 hr. periods of light and dark. During the first period of darkness figured, a locomotor phase began and the animal moved across the stone to which it had attached itself. The greater frequency of the contractions of the column during this phase is conspicuous.

As in the phase of activity of the column associated with feeding, we have in locomotion a co-ordinated pattern of activity which can appear in the apparent absence of any stimulus. It may follow a stimulus, but the phase may endure for hours after its cessation. The stimulus initiates the phase, but its continuation is not necessary for the phase to be maintained. The threshold of the phase varies and can be affected by a number of factors, such as food which lowers the threshold and light which raises it. These features are characteristic of phasic activity.

PHASES OF EXPANSION AND CONTRACTION

That many sea-anemones exhibit alternating phases of prolonged general expansion and retraction is well known (Parker, 1919). This alternation has sometimes been correlated with the sequence of night and day, whilst other workers have stressed its relation to the tide. Parker has shown that there is a well-marked rhythm frequently to be observed in *Metridium marginatum*, and has related this to the daily rhythm of light and darkness. As he points out, *Metridium* is undoubtedly sensitive to light, and we can fully confirm this, though, as Cotte (1922) remarks, the responsiveness of individual actinians to light is very variable.

The direct action of light on a *Metridium* which has been in darkness causes a slow contraction of the parietal musculature. This response is local if the illumination is local, resulting in contraction of that sector of the parietal wall on which the light falls. It thus differs from the generalized parietal contraction caused by electrical stimulation (Hall & Pantin, 1937). If the illumination is general and prolonged, the parietal contraction is followed in unfed anemones by a withdrawal of the tentacles, closing of the sphincter, and further shortening of the column; the animal

then maintaining a phase of prolonged contraction during which inherent activity takes place with the anemone in a much greater state of average tone.

In the following experiments we are primarily concerned with the influence of light and darkness on rhythmic phases of contraction and of expansion of the order of 12 hr. duration. Hence environmental conditions had to be controlled as far as was practicable for periods of days or even weeks. Freshly collected anemones were first kept for a week without food. Then each, after it had become attached to a stone, was placed separately in a glass aquarium tank holding about 15 l. of clean sea-water. This was kept gently circulating and aerated by compressed air. The glass tanks were placed in a cellar hewn out of rock, where extraneous mechanical disturbances were minimal, illumination could be controlled, and temperature variations were slight (varying by only 1–2° C. over periods up to 6 weeks). A stimulus that could not be avoided was the attachment of the recording thread to the anemone. This was done by a fine glass hook. The hook was very gradually extruded from the tissue by a process of local destruction of the tissue on one side

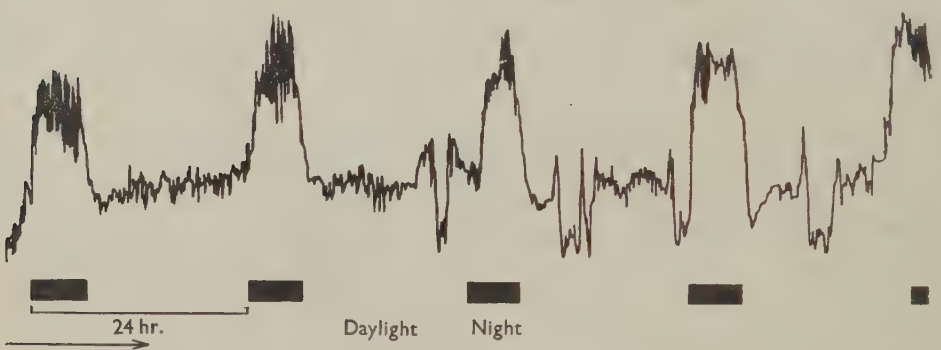


Fig. 7. Vertical activity of *Metridium* exposed to natural daylight and darkness. Well-marked light-controlled rhythm is shown by this specimen.

and regeneration on the other, so that over long periods re-hooking was necessary. However, a single 'hooking' might last for a week or more.

Sometimes hooking initiated locomotion on the succeeding night. But in general it caused no more than a temporary contraction, and effects seen in the records could all be confirmed in normal animals without hooks attached. Breaks in the tracings shown in Figs. 8, 9 and 11 are periods not recorded because the hook had been sloughed off. In nearly every instance the anemone remained in an apparently healthy condition, and the water remained clean throughout the period of the record.

Illumination was effected by a 150 W. clear glass light bulb at distances ranging from 160 to 225 cm. from the anemones. During illumination, the recorded contractions could be related to the state of the animal by direct observation. During continuous darkness this naturally could not be done. In some cases the animals were examined occasionally during darkness with the aid of a red hand-light rendered as dim as could be used by a dark-adapted observer. In one case, and only

one, was there any indication that this extremely weak illumination affected the animal in any way. One animal showed a slight contraction on two occasions when exposed to the dim hand-light.

Many, but not all, individuals of *Metridium* show a marked diurnal phasic rhythm, not only in their natural habitat but also when placed in an aquarium exposed to daylight. Fig. 7 shows a typical case. During the night the continual inherent activity of the animal took place about a phase of expansion, and by day about a phase of retraction. The aquarium was in this case set up exposed to daylight in a north-facing window of the Marine Biological Laboratory, Plymouth. Such a clear rhythm as this is moderately common, though by no means universal.

The phasic rhythm of animals similar to that shown in the experiment illustrated in Fig. 7 is controlled by light. Fig. 8 shows part of the tracing of the activity of a starved *Metridium*, recorded over a period of 48 days in all. The temperatures recorded twice daily during this very long experiment ranged from 16·2 to 16·8° C. The aquarium could be illuminated when desired by a 150 W. electric bulb 185 cm. from the anemone.

For the first fortnight the last 4 days of which are recorded in Fig. 8, the light was switched on at 9 a.m. and off at 9 p.m. (G.M.T.). There was thus alternate light and darkness approximately corresponding to the outside day and night. There is an evident light-controlled diurnal rhythm of contraction and expansion. The anemone was then left in darkness for 22 days and nights. Finally, alternating 12 hr. periods of light and darkness were recommenced, but now with illumination during the night and darkness during the day. The corresponding reversal of the rhythm during this last phase is clearly shown and indicates the directness of the relation of the alternations of phase to the state of illumination. Such results show that *Metridium* may possess a rhythmic alternation of its phases of extension and retraction that can be controlled by light, with all other environmental factors maintained constant.

But the experiment illustrated in Fig. 8 shows another feature of great interest. During the intermediate period, the anemone was maintained in complete darkness and the external conditions were varied not at all. Nevertheless, the general activity was as great as during the periods of daily illumination, and moreover changes of phase are still evident. The phases of contraction and expansion are at first somewhat irregular, but they presently develop a rough regularity with a period somewhat greater than 24 hr. The rhythm is by no means exact, and the phases soon get quite out of step with the previous periodic illumination or, for that matter, with the sequence of external day and night.

Inherent changes of phase in *Metridium* under constant external conditions are common. But they do not always show so clear an approximation to a regular rhythm as is to be seen in Fig. 8. Usually they are very variable and are often of short duration. Sometimes for long periods there is no evident change of phase. Fig. 9 shows a record of a *Metridium* which, after exposure to alternating periods of light and darkness, was then kept in continuous darkness for several days. During the first 30 hr. of darkness this individual showed no phase changes. After this the

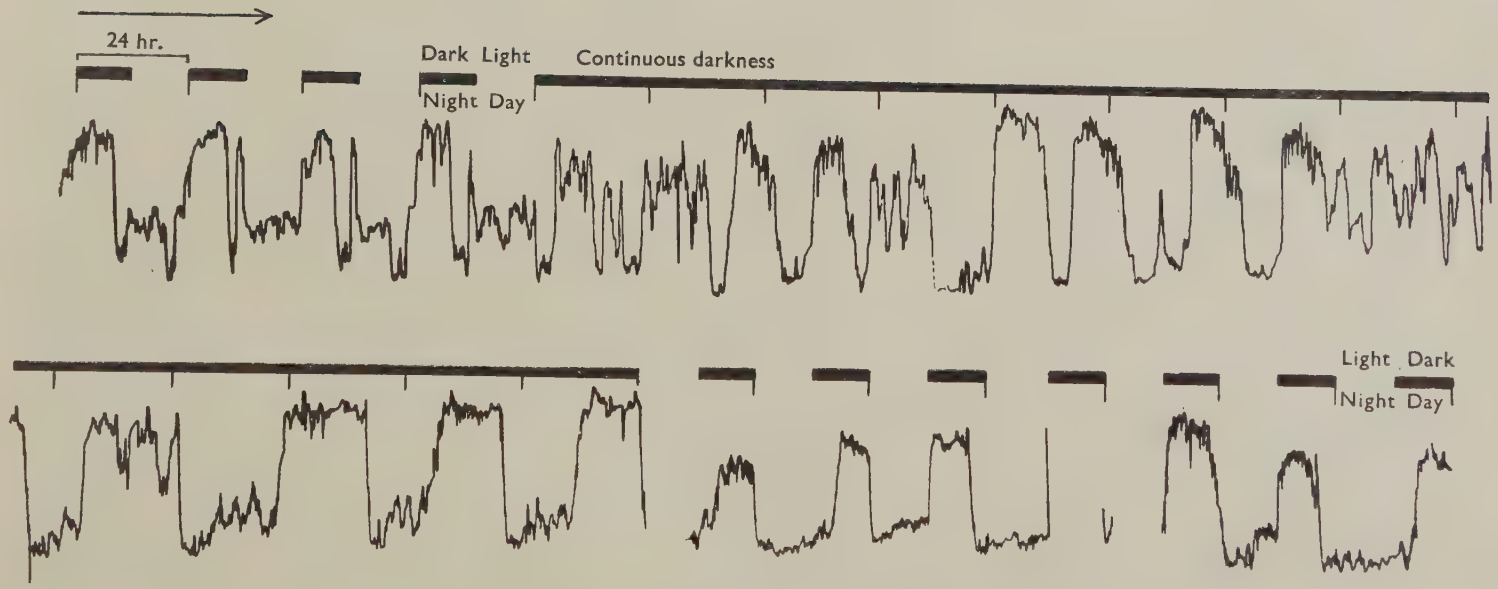


Fig. 8. Portions of 48-day kymograph record of activity of *Metridium*. Alternating 12 hr. periods of light and dark at beginning correspond to day and night, at the end are reverse of this. Note light-controlled rhythm. During intermediate period of prolonged darkness there develops an approximate phasic rhythm unrelated to external diurnal or tidal rhythm.

activity alters its character and irregular alternations of phase occur; but it is far from a regular rhythm.

Though inherent changes of phase in constant conditions are usually irregular, phase changes are very commonly set into a rhythm by periodic stimuli. In Figs. 7 and 8 we see how periodic illumination can control rhythmic phase change. Other stimuli as well as light may be effective in controlling phase changes. Parker (1919) showed in *M. marginatum* that water movement could cause expansion, and that through this factor the tides might act as a pace-maker for expansion and contraction, as well as light. Fig. 4 also makes it clear that even periodic exposure to appropriate chemical stimuli might enforce periodic expansion.

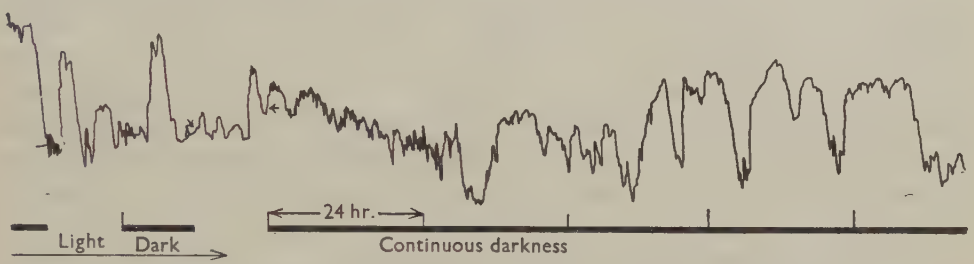


Fig. 9. Kymograph tracing of vertical body-wall activity, showing a change in character of phasic activity during a prolonged period of constant, dark conditions.

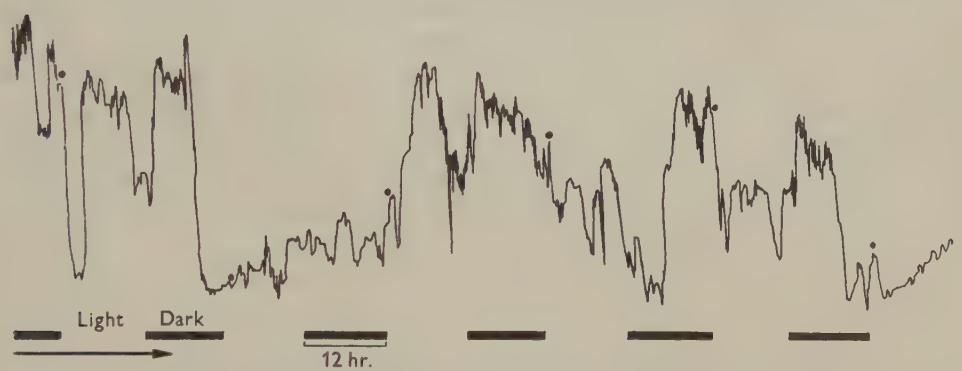


Fig. 10. Record of activity of specimen showing irregular phase changes unrelated to light and dark. Dots mark points when light was turned on.

But the connexion between stimulus and change of phase is not simple. Regular stimuli may not always succeed in controlling phase change. We may contrast the experiment shown in Fig. 8 with that of Fig. 10. In the latter the animal shows irregular alternations of phase although exposed to regular alternate 12 hr. periods of light and darkness. At the onset of each light period the animal gave some degree of contraction, but its extent was variable and the contracted phase was not maintained. The responses to light are marked by dots on the tracing. In this case the light stimuli, though effective in causing immediate contraction, did not succeed in controlling phase change. The accessibility of phase change to stimulus is as

variable in the case of periodic light stimuli as we have seen it to be in the case of food stimuli; and not only do different individuals vary in this respect, but the same individual varies on different occasions.

The phases of contraction during periodic illumination are not simply direct responses of a passive animal to light. The stimulus is not exciting an inert system. In the absence of stimulation, phase changes of an irregular or semi-rhythmic character take place inherently, and a periodic stimulus appears to act by controlling this inherent rhythm. The effectiveness of the stimulus in initiating a contraction phase varies with the state of the animal and the illuminating stimulus may or may not be effective in controlling rhythmic expansion and contraction. But when it is effective it appears to act by setting the pace of the inherently occurring phase. Examination of Fig. 8 shows that even when a good rhythm is established, the inherent rhythm may from time to time break free of the pace-setting stimulus. The fact that the periodic illumination appears to be setting the pace for an inherent alternation of phase is also to be seen in a curious effect not infrequently observed with well-established light rhythms: the phase change may anticipate the recurring stimulus, so that the animal passes into the contracted phase just before the daily illumination commences. This recalls various claims that *Actinia equina* may 'anticipate' tidal stimuli (Parker, 1919).

Fig. 8 plainly shows that on occasion *Metridium* can exhibit inherent periodic changes of phase which may approximate to a rhythm in the absence of any rhythmic external stimulation. Even well-established inherent phasic rhythms vary widely in frequency, and though sometimes exhibiting a period of the general order of 24 hr. there is no close approximation to this period. But the existence of such more or less rhythmic changes of inherent origin may perhaps account for recorded cases of apparent persistent night-and-day and 'tidal' rhythms, such as the tidal rhythm recorded by Bohn (1906, 1909, 1910) in *Actinia equina*, which was said to be maintained in phase with the tides for 2–3 days, or even longer, after removal of the animal to constant conditions.

Many have doubted the existence of such rhythms, notably Piéron (1908) on *Actinia*, Parker (1919) on *Sagartia luciae*, and Gee (1913) on *Cribrina*. We can confirm the absence of any exact rhythm persisting in phase with either the tidal or the daylight cycle. *Metridium* observed under constant conditions in the laboratory immediately after their removal from the intertidal zone showed no relation in their subsequent phasic activity to the external tidal rhythm. Similarly, fifteen specimens of *Actinia equina*, which were collected from their upper intertidal limit and immediately placed under constant conditions in a dimly lit cellar, showed no trace during the following 3 days of a persistent tidal rhythm in the absence of the direct influence of the tide.

Remarkably accurate persistent rhythms have been reported from organisms widely separated in the animal kingdom. Gunn (1940) records persistent and exact diurnal rhythmic activity to be maintained in the cockroach *Blatta orientalis* for upwards of a week in constant conditions. Among Protista, Bracher (1937) records the rhythmic migration of the estuarine flagellate *Euglina limosa*. A tidal rhythm is

maintained for 3 days under constant laboratory conditions. Likewise, Fauré-Fremiet (1948) records the remarkable tidal excystments of the ciliate *Strombidium oculatum*, which are maintained for 2–3 days in constant laboratory conditions. *Metridium* and *Actinia* certainly do not possess 'internal clocks' capable of maintaining such accurate rhythms: nor are they in some mysterious way *en rapport* with rhythmic events in the outside world from which they are experimentally isolated. But the fact that Actinians removed to constant environmental conditions may sometimes develop inherent phase changes of the general order of 24 hr. might well lead occasionally to the impression of a persistent rhythm.

Parker (1919) questioned the existence of any persistent rhythms in phase with the tides or with previous daily illumination. But he advanced as evidence against the existence of such rhythms the fact that specimens of *Metridium* he kept in darkness remained continuously expanded for some 36 hr., and he implied that phase change only occurred as the direct result of a stimulus. With this last implication we cannot agree. Inherent phase change under constant external conditions certainly takes place, though the extent of its development is very variable in different individuals, and 36 hr. is often too short a period for its unequivocal manifestation.

From our experiments we conclude that the phases of contraction and expansion accompanying diurnal illumination resemble the phases we have discussed in connexion with feeding and locomotion. The threshold for the initiation of the phase varies in different animals. In many, a phase of contraction is easily initiated by the stimulus of illumination, in others it is not. The threshold may be so low that the phase is initiated spontaneously under constant external conditions. The new and interesting feature of these phases of expansion and contraction is that in those anemones in which they occur spontaneously there may on occasion develop a roughly rhythmic alternation of the two phases with a frequency which is sometimes of the general order of that of night and day, though not necessarily in phase with it. Under these conditions spontaneous phase change can often be set in step with a regular external stimulus such as that of daylight and darkness to give a well-marked diurnal rhythm.

THE INTERACTION OF PHASES

Though the pattern of activity which constitutes a phase may primarily concern one particular system of effectors, such as the muscles of the pedal disk, all parts of the animal are to some extent involved in them. Consequently, two separate phases may reinforce or may conflict with one another.

The most striking example of conflict of phases is seen in the effect of feeding on the light-controlled rhythm. If a *Metridium* which has been regularly closing and remaining contracted during light and expanded during darkness is given a meal (e.g. half a *Mytilus*), its behaviour is modified for several days afterwards. When illuminated after feeding, it still gives initially the usual parietal shortening. But then, instead of assuming an enduring phase of contraction, the column elongates again notwithstanding the light, and its disk usually remains expanded throughout. The food has in some way temporarily abolished the capacity to retain a state of

phasic contraction during prolonged illumination. The inhibitory effect of the meal persists for about a week, after which phasic retraction to periodic illumination gradually returns.

Not only may phasic retraction to light be inhibited for several days after a meal but occasionally some of its features even appeared to be reversed. In some specimens for the first day or so the sphincter, which is closed in complete retraction, actually opened more fully when first illuminated.

This inhibition by other phases, of phasic retraction to periodic illumination is shown in Fig. 11. For the first 3 days of the record the animal shows a clear light-

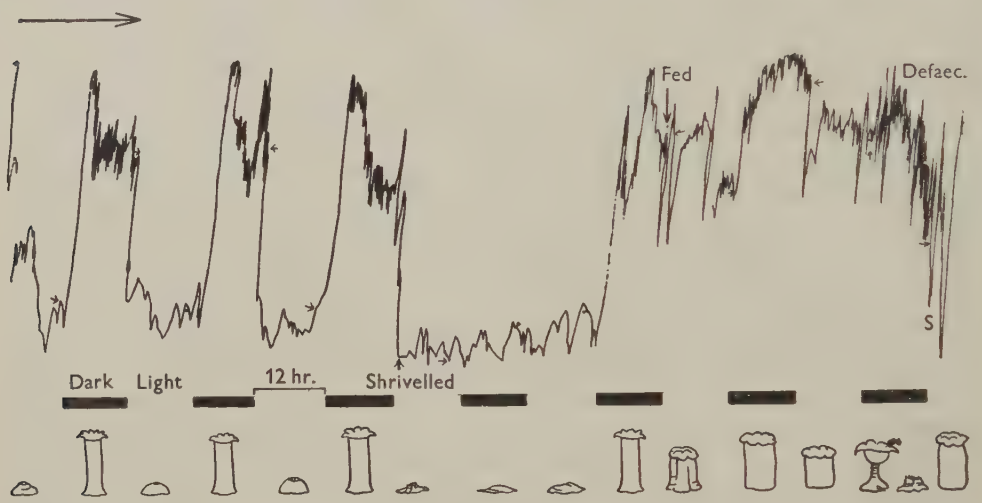


Fig. 11. Kymograph tracing of activity of specimen showing marked light-controlled rhythm prior to feeding, which is largely abolished by a meal. Figures below show appearance of anemone at successive points on the record. For full explanation see text.

controlled phasic rhythm. At the beginning of the fourth day the animal passed spontaneously into the shrivelled state, and thereafter remained contracted for 36 hr. The failure to expand during the fourth night may possibly be due to repeated examination with dim red light. At the beginning of the fifth night, however, the animal expanded. During that night it accepted a meal. Thereafter the animal remained for 48 hr. in a predominant phase of expansion. The three periods of illumination succeeding the meal began 80 min., $24\frac{1}{2}$ and $48\frac{1}{2}$ hr. after it. On each of these three occasions, the switching on of the light caused some contraction; but this was not maintained, nor did the sphincter maintain closure.

About 36 hr. after the meal the expanded animal passed into a phase of great activity associated with defaecation (Defaec.). Finally after defaecation, soon after the beginning of the last day recorded, the animal shrivelled (S) and afterwards rapidly re-expanded in a greatly inflated condition. The immediate sequence of events following the meal is essentially the same as that seen in Figs. 2 and 3. The predominant expansion associated with the feeding phase in Fig. 11 has obliterated the earlier light-controlled rhythm and has reduced the response to illumination to

a variable temporary contraction. Phases may therefore block each other, much as we see in the case of incompatible reflexes.

A complete extension phase induced by food is incompatible with a retraction phase to light. But the causes of inhibition of a phase are not always obvious. There is no clear reason why light should inhibit locomotion; though we may note that light causes contraction whilst food, which may excite locomotion, causes expansion. There may be some correlation between the phase of locomotion and the phase of expansion. Why this should be is not clear, though peristalsis of the column, which plays a significant part in locomotion in *Metridium*, is somewhat more evident during phases of expansion.

DISCUSSION

Parker (1919) came to the conclusion that an Actinian was an animal whose internal state approximated to one of general uniformity and that the changing environment calls forth various responses without seriously disturbing the internal equilibrium. He contrasts this with the condition in higher animals in which response is novel and diverse, and in which much of the behaviour has that character which leads us to describe it as spontaneous. Our observations show that this view of Actinian behaviour is too simple.

We showed in the previous paper (Batham & Pantin, 1950*b*) that *Metridium* is in a state of continual and varying activity which is not a series of direct responses to external stimuli. The activity is a character of the unstimulated animal, and we have termed it inherent. The activity may be truly spontaneous, or it may involve internal stimuli though, if so, these must be more complex than those of a simple chain reflex. We see in the present paper that the pattern of this activity can change through a series of phases. Like the activity itself, a phase is an inherent character which may appear in the externally unstimulated animal; the animal will change from one phase to another under apparently constant environmental conditions. Whether the change of phase is truly 'spontaneous' or whether it depends upon internal stimuli is not yet certain. It is well to bear in mind that in the end this distinction may merely depend on whether or not there is an internal mechanical link in the cycle of events leading to apparently spontaneous activity. We shall return to this question in a later work.

Like simple reflex responses, phase changes may be induced by external stimuli. But as we have seen, the relation of a phase to a stimulus is much more complex and in many ways different in character. In particular, the stimulus initiates the phase rather than maintains it; and the whole pattern of activity of the phase is independent of the stimulus. It may be initiated by various external stimuli or apparently initiate itself. While some of the responses of *Metridium* to stimuli have the character of simple reflexes as in the retractor response, others, such as those involved in phasic activity, are more complex, more variable and depend on the state of the animal. These are the very characteristics which Parker so truly noted in the behaviour of the higher animals but supposed to be relatively insignificant in Actinians. It was precisely this variability of behaviour and the dependence of response upon the

state of the organism which was stressed by Jennings (1915). The figures in this paper, in fact, give objective records of the existence in *Metridium* of 'physiological states' such as Jennings showed to be characteristic of all organisms from Protista upwards including Coelenterates.

Inherent phasic activity is an integral part of the behaviour patterns of *Metridium*. In the first place, muscular action due to inherent activity balanced against the coelenteric pressure is responsible for the shape of the animal (Batham & Pantin, 1950*a*). Secondly, the activity exhibited during a phase is behaviouristically significant. The elongation and swaying movements which follow temporary exposure to food solution seem quite evidently purposive in that they increase the chance of discovering a food object, the presence of which might be signified by the original stimulus. We see here inherent activity playing a significant part in a behaviour pattern, and moreover it does so in a peculiar manner. Actions such as swaying are behaviouristically relevant not so much to the original stimulus as to a future event, the capture of a solid food object, the probability of which is increased by sweeping the ground in the neighbourhood. Such activities seem so clearly purposive, and seem so clearly directed towards a goal in a manner we ourselves can understand, that earlier workers such as Gosse (1860) inferred the existence of consciousness and will in these animals. Such an assumption is unnecessary; the properties of phasic activity are completely open to mechanistic analysis. But it is idle to neglect the fact that the mechanisms involved in phasic activity are of a much higher order of complexity than simple and direct reflex responses such as have been demonstrated by earlier workers (Parker, 1919; Pantin, 1935). Some responses, such as the retractor response and the transfer of food by the tentacles to the mouth are undoubtedly simple and direct, and their physiological analysis is in parts tolerably complete. The fact that these activities are rapid makes them easily perceived, whereas the more complex phasic activities of *Metridium* are so slow that they are commonly overlooked. Consequently, unless prolonged observation is made we may easily receive the false impression that Actinian behaviour consists of simple direct reflex responses to external stimuli by a passive animal.

But even the direct reflex responses of Actinians are not quite so simple as would at first appear. Simple patterns of nerve impulses can be set up by electrical stimuli which call up feeding, retraction and so on. But when we consider these responses as they are initiated by natural stimuli, it is evident that there is some degree of complication on the sensory side, which is 'short-circuited' by direct electrical stimuli. The threshold of chemical stimulus required to produce reflex capture of food by the tentacles varies greatly with the state of the animal (Pantin & Pantin, 1943). After a meal the animal is much less sensitive to food than before (Parker, 1919). This may be due to sensory adaptation. But a meal also appears to raise the threshold for mechanical stimulation (Pantin, 1935). And even Actinians that have been starved together in a tank show great and varying changes of thresholds for food capture by the tentacles. Thus, in an experiment, a piece of mussel flesh was placed on the tentacles of each of twenty-four *Metridium* previously starved for at least 3 weeks. In thirteen the food was accepted at the first offer, in ten of the others

it was accepted between the second and fifth offers, whilst one refused to feed. Similarly, the time taken for food to be ingested varies greatly. In a series of eleven observations food placed on the oral disk required in different cases from 3 min. to 1 hr. 45 min. to be passed into the coelenteron.

Even for direct responses such as food capture and retraction there are factors as yet unidentified which vary the sensory threshold of the response with the state of the animal. It is to be noted that one of the anemones which failed to accept food did so because the threshold for mechanical stimulation had temporarily become so low that even the light contact of a food object with the tentacles brought about retraction.

Nor are the direct responses of Actinians influenced only by changes in sensory threshold. Chemical changes in the medium can greatly modify the extent of the sphincter response in *Calliactis* following electrical stimuli. Ross & Pantin (1940) showed that excess magnesium depressed the response, whilst excess potassium augmented it. Chemical factors may influence facilitation and other properties of the excitation system. In Vertebrates, Bozler (1941) showed that the excitability of mammalian uterus varies greatly according to endocrine conditions. Excitability is very low during anoestrus and reaches its height during oestrus. The previous injection of oestrogenic substances causes the muscle to pass from a condition of low excitability and local response to one of high excitability and total response. There are many similar cases of endocrine influence upon response. These examples suggest the possibility that in Actinians both direct responses and inherent activity may conceivably be influenced by endocrine or other chemical changes in the tissues. Such changes might indeed be primarily responsible for changes of phase. But on this point we have at present no evidence.

In conclusion we would draw attention to the very considerable complication of response mechanism in Actinians, notwithstanding their low grade of morphological organization. We are, in fact, led to consider the direct reflex responses as secondary simplifications to meet specific purposes, rather than as examples of an elementary unit of all behaviour patterns. As Jennings (1915) showed, many supposed characteristics of behaviour of the higher animals are already present in the Protozoa. The varied phases of *Metridium* call to mind not only the more complex behaviour of the higher animals, but also the physiological states described by Jennings in *Stentor*. There is similar variation of character and threshold. Moreover, we already have in the Protista clear evidence of inherent rhythmic activity which may vary in frequency from ciliary or flagellar action to the diurnal and tidal rhythms of *Euglena* and *Strombidium* already referred to. Inherent activity and phasic change are characteristic of all grades of organism and, since they are found in Protozoa as well as Metazoa, it is evident that their mechanisms can be achieved in more than one way. If we find on occasion that they appear to be absent, and that behaviour seems to consist of simple reflexes, we should perhaps rather look for some evolutionary reason for the suppression of inherent activity than consider that we are dealing in such cases with primitive nervous machinery.

SUMMARY

1. In constant environmental conditions *Metridium senile* exhibits continual slow inherent activity. The pattern of this activity varies in character from time to time. These different patterns of activity have been termed 'phases'. A particular phase may endure for hours on end and then rather quickly give place to another phase.

2. A change of phase may be initiated by certain stimuli. Ingestion of food initiates a sequence of phasic changes involving expansion of the disk and elongation, followed by swaying, parietal contraction, distension, defaecation and 'shrivelling'. Each of these phases has its own pattern of activity. Not all may be exhibited by any one animal, or by the same animal at different times. A similar sequence of phases to those induced by solid food may be initiated by mere temporary exposure to filtered food solution.

3. In contrast with the direct responses, such as the retraction reflex, the stimulus does not directly maintain a phasic response; it merely initiates a new phase, and the activity pattern of this new phase is maintained long after (even hours after) the initiating stimulus. This relation of phase change to stimulus may be evident after electrical stimulation as well as after exposure to food or other stimuli.

4. Phase changes also differ from simple reflexes in that the threshold for their initiation varies enormously in different animals and in the same animal at different times. Also the threshold may sometimes be so low that the phase occurs spontaneously in the absence of evident external stimuli. Changes of threshold cannot be adequately accounted for by sensory adaptation. The stimulus apparently acts by releasing a complex activity pattern (the phase) which is so far independent of the stimulus that it may appear spontaneously.

5. Locomotion is another phasic activity. It is a complex co-ordinated activity pattern. It may be initiated by various stimuli. The threshold varies at different times and in different animals. It may take place spontaneously in the absence of evident external stimuli. The threshold of this phase is lowered after feeding and raised by illumination.

6. Alternating phases of expansion and contraction frequently occur in *Metridium*. Their relation to diurnal and other rhythms is discussed. Daily illumination can often initiate and control regular daily phases of contraction. This is true both of daylight and of periodic exposure to artificial light. As with other phase changes, the threshold varies greatly. In some cases each periodic illumination may only induce a brief temporary contraction and fail to control phase change.

7. In complete darkness and constant environmental conditions, alternating phases of expansion and contraction may still take place. These may be irregular, but sometimes they may assume a very rough rhythm. When present, this rhythm does not keep in step with previous daily stimuli, nor with current external changes of day or night, nor with other environmental rhythms. The unstimulated animal thus possesses an inherent tendency to alternating phase change which may approach a rhythm. It appears that periodic stimuli, such as daily illumination, act by 'setting the pace' of this inherent alternating phase change.

8. Different phases which involve the same groups of effectors may reinforce or may conflict with one another.

9. Our experiments show that continual and varying patterns of inherent activity play an important part in the behaviour of *Metridium*. The behaviour is not simply a succession of direct reflexes to stimuli acting on a passive animal. Such direct responses are, however, more easily observed because phasic activity is extremely slow. Phasic activities play an essential part in behaviour patterns such as food capture. They are often behaviouristically relevant to a future possible event rather than to a past stimulus; as when sweeping and swaying movements increase the chance of finding food.

The relation of phases to the 'physiological states' of Jennings is noted.

Much of this work was done at the Marine Biological Laboratory, Plymouth, to the Director and staff of which we are most grateful for the many facilities given us. Part of the work was done during the tenure by one of us (E.J.B.) of a Shirtcliffe Fellowship of the University of New Zealand. We wish to thank the Department of Scientific and Industrial Research for a grant for the development of a special research which enabled this work to be concluded.

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THE FERTILIZATION REACTION IN THE SEA-URCHIN EGG. THE EFFECT OF NICOTINE

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(*Received 7 March* 1950)

(With Plate 12 and One Text-Figure)

INTRODUCTION

After insemination of unfertilized eggs of *Psammechinus miliaris*, a change in cortical structure is propagated over the egg surface. A quantitative examination of this phenomenon (Rothschild & Swann, 1949) raised the following questions, requiring further experiments. First, is the observed cortical change the block to polyspermy, or is there a more rapid and so far undetected block to polyspermy as suggested by Gray (1931)? Secondly, is the cortical change directly or indirectly caused by the diffusion of some substance from the sperm head, round the egg surface or through the cytoplasm? The second question is not considered here, though some calculations relating to it have been published elsewhere (Rothschild, 1949).* The first question is the subject of this paper.

Hertwig & Hertwig (1887) discovered that nicotine, among other substances, induced polyspermy in sea-urchin eggs. An investigation of the action of nicotine involves consideration of the following possibilities. If nicotine increases the mean speed of a sperm suspension, the number of sperm-egg collisions will be greater than under normal conditions, thus increasing the incidence of polyspermy. Secondly, if nicotine retards or suppresses the block to polyspermy, and the observed change is the block to polyspermy, it should be possible to detect this action of nicotine. Finally, the suggestion has been made that not every sperm-egg collision is successful in the sense that fertilization follows. Nicotine may have a chemical effect on the egg surface such that the probability of a successful sperm-egg collision is increased, with concomitant changes in the incidence of polyspermy.

The experiments described in this paper were designed to investigate these questions. Eggs, with and without jelly, and spermatozoa of *P. miliaris* were used.

The fertilization reaction and the movements of spermatozoa were recorded by the dark-ground photography methods described elsewhere (Rothschild & Swann, 1949), while sperm densities were measured with the photoelectric absorptiometer (Rothschild, 1950).

* There is a misprint in this paper on p. 179, where the approximate figure 10^3 for the molecular weight should be 2.10^4.

RESULTS

Incidence of polyspermy at different sperm densities. Nicotine-treated and control eggs were placed in different sperm suspensions of the same density. At the time of the first cleavage the numbers of unfertilized, monospermic, polyspermic and cytolysed eggs were counted. The results of an experiment of this type are given in Table 1.

Table 1. *Effect of nicotine on incidence of polyspermy in eggs of* Psammechinus miliaris

Eggs treated with nicotine, N. Eggs not treated with nicotine, C. *Nicotine treatment:* unfertilized eggs placed for 5 min. before fertilization in 1/2000 (v/v) nicotine in sea water. Figures in brackets are percentages. $T°$ C., 17. 7 July 1949.

No. of sperm/ml.		Unfertilized eggs	Monospermic eggs	Polyspermic eggs	Cytolysed eggs	Doubtful
$9{\cdot}07 \times 10^4$	C	36 (9·3)	340 (87·9)	1 (0·3)	9	1
$9{\cdot}07 \times 10^4$	N	30 (6·4)	326 (69·8)	109 (23·3)	2	0
$1{\cdot}36 \times 10^6$	C	5 (1·4)	322 (92·8)	5 (1·4)	15	0
$1{\cdot}36 \times 10^6$	N	2 (0·4)	149 (32·6)	289 (63·2)	17	0
$2{\cdot}18 \times 10^7$	C	12 (2·9)	351 (85·8)	23 (5·6)	22	1
$2{\cdot}18 \times 10^7$	N	1 (0·2)	45 (10·9)	350 (85·0)	16	0
$3{\cdot}48 \times 10^8$	C	0 (0)	253 (63·4)	118 (30·0)	28	0
$3{\cdot}48 \times 10^8$	N	1 (0·2)	3 (0·7)	437 (98)	5	0

The expected number of successful collisions while the block to polyspermy is being propagated can be calculated from the ratios of monospermic to polyspermic eggs (Text-fig. 1).*

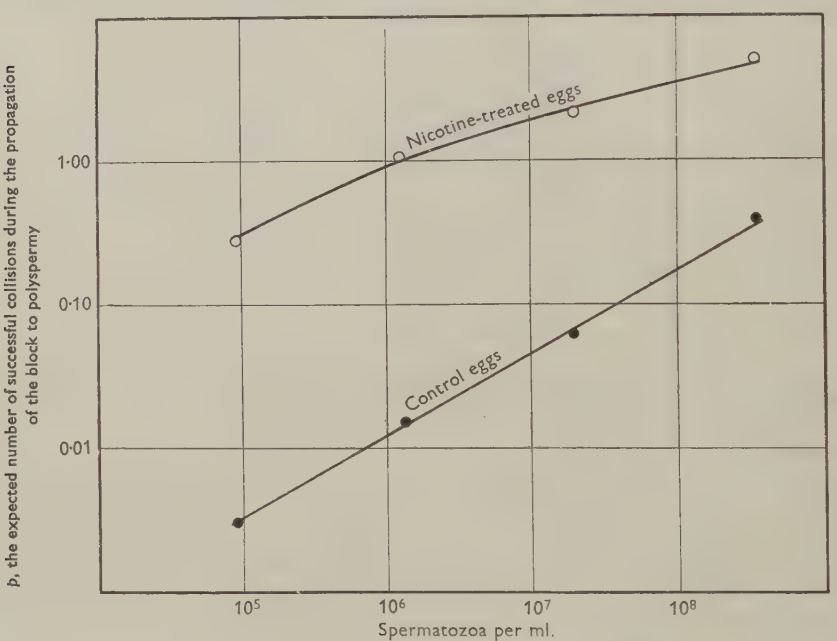
The expected number of successful collisions during the propagation of the block is evidently related to sperm density in an orderly but not a linear way. If the sperm density is increased by a factor of 1000, the increase in the expected number of successful collisions is about 12-fold for nicotine-treated eggs and about 60-fold for untreated eggs. The effect of nicotine is to increase the expected number of successful collisions at all sperm densities, the increase varying from about 100-fold at 10^5 sperm/ml. to about 13-fold at 10^8 sperm/ml.

The existence of an orderly relationship between the expected number of successful collisions and sperm density means that nicotine does not completely suppress the propagation of the block to polyspermy. But the fact that the relationship is not linear suggests that, as predicted, the assumptions involved in treating the collisions between spermatozoa and eggs in terms of the bombardment of a particle of a known radius by gas molecules are unlikely to be justified over wide ranges of sperm densities. One reason concerns the possibility that spermatozoa do not swim at the same speeds at different densities (Gray, 1928). Sperm tracks in the neighbourhood of a drop of undiluted semen, Pl. 12*a*, can be compared with those in a sperm

* The treatment involves the assumption of a Poisson distribution. If it were shown that the expected number of successful collisions and the conduction rate of the block to polyspermy varied from egg to egg, a different distribution would be applicable. Deviations from the Poisson distribution would also suggest this possibility.

suspension of density 10^5/ml., Pl. 12*b*. The evidence in support of a difference in sperm speeds in dense as opposed to dilute suspensions is not strong. The presence of jelly round the egg has little effect on the speeds of spermatozoa moving in it, though there is a tendency for less spermatozoa to be found inside the jelly than on or near its outer surface. Before the problem of the non-linearity of the sperm density-probability relationship can be explained, more detailed observations of the behaviour of spermatozoa near an egg are needed.

The effect of nicotine in increasing the expected number of successful collisions during the period of propagation of the block to polyspermy means that the block itself is slowed down, or that the chance of a collision being successful is raised.



Text-fig. 1. Relationship between p, the expected number of successful collisions during the propagation of the block to polyspermy, and sperm density. Using the Poisson distribution, the proportions of monospermic and polyspermic eggs will be e^{-p} and $1-e^{-p}$.

The effect of nicotine on the speeds of spermatozoa and on the rate of propagation of the cortical change. Pretreatment of unfertilized eggs with nicotine has no effect on the mean speeds of spermatozoa. Furthermore, neither the conduction velocity nor the appearance of the propagated change are significantly altered by nicotine. The effect of nicotine in inducing polyspermy can therefore have one of two explanations. Either the block to polyspermy has a much higher conduction velocity than that of the observed cortical change, and *this* block to polyspermy is slowed up by nicotine; or, if the observed cortical change is the block to polyspermy, there must normally be a low probability of a successful sperm-egg collision, which is greatly increased by nicotine. The experiments in the next section constitute an attempt to decide between these alternatives.

The effect of nicotine on the time between insemination and fertilization. If there is a low probability of a successful collision we should expect considerable scatter or dispersion in the 'fertilization' times within a sample of eggs which have been inseminated at $t=0$. If nicotine increases the probability of a successful collision, the dispersion and the mean time at which 'fertilization' occur should be reduced. A series of experiments were carried out in which eggs from the same female were added to sperm suspensions of known densities. The experiments were done in pairs, using nicotine-treated and untreated eggs. Immediately after insemination the eggs were transferred to the microscope slide and dark ground cinematography was begun. The times at which eggs first showed any cortical brightening (i.e. 'fertilization') were determined. The results of the experiments are shown in Table 2.

Certain conclusions may be drawn from these experiments:

(1) No egg shows a cortical change in less than about 20 sec. after insemination.

(2) Though the mean times of treated samples are somewhat less than those of untreated samples, the difference is not statistically significant.

(3) There is a significant reduction in dispersion as a result of nicotine treatment.

If there is a high-speed block to polyspermy and a high probability of a successful collision, the alternative to a low-speed (20 sec.) block to polyspermy and a low probability of a successful collision, the delay of 20 sec. before any egg shows the cortical change must be attributed to a latent period during which no visible morphological changes occur, even though a spermatozoon has activated the egg. If this hypothesis is correct, all eggs will be fertilized in the first few seconds after insemination, and the considerable dispersion of the times at which cortical changes occur must be attributed to variations in the lengths of this latent period. The reduction of this dispersion in nicotine-treated eggs would have to be explained by the somewhat improbable postulate that this treatment produces some reduction in the variability at which the rates of the chemical and physical reactions responsible for the latent period take place.

If, alternatively, the observed cortical change is the block to polyspermy and there is a low probability of a successful sperm-egg collision, the 20 sec. delay in appearance of the cortical change and the lack of reduction in the mean time at which the cortical change appears are difficult to explain without making further assumptions that have no experimental justification. The reduction in dispersion is, however, consistent with this hypothesis.

Direct observations. Rothschild & Swann (1949) briefly discussed the direct evidence that spermatozoa may collide with an egg without fertilizing it. Moser (1939, p. 428) refers to spermatozoa bombarding the surfaces of eggs of *Arbacia punctulata* 'to the extent that momentary indentations may be seen in the periphery of the eggs'. We have made similar observations using the eggs of *P. miliaris*. This evidence tends to support the hypothesis that the conduction velocity of the block to polyspermy is low and that the probability of a successful sperm-egg collision is low; but there is a possibility that the observed unsuccessful collisions in fact take place after a spermatozoon, which has not been seen, has activated the egg.

Table 2. *Effect of nicotine on time at which cortical change develops after insemination*

C, controls; N, nicotine-treated eggs. The figures in columns C and N refer to the film frame numbers, the number of frames per second being 1·5. The experiments are tabulated in ascending order of sperm densities. *T* ° C., 17.

Spermatozoa per ml.	Exp. no.	C		N	
		Frames after insemination	Mean	Frames after insemination	Mean
$1{\cdot}3 \times 10^5$	1	43 86 101	76·7	46 48 67	53·7
5×10^5	9	47 94 113 140	98·5	72 84 85 107	87·0
10^6	8	61 73 75	69·7	39 44	41·5
2×10^6	2	47 52 54		40 45 59	
	3	44 44 45 45 53	48·5	57 61 62	49·4
	7	37 38 52 71		38 40 42 50	
4×10^6	4	29 39 40 45 55 62	45·0	33 43 48	40·0
	5			31 45	
5×10^6	10	—	—	48 54	
	11	—	—	42 43 44 45	
	12	—	—	43 50 51	47·7
	13	—	—	48 49 54 56	
	14	—	—	43 44 49	
8×10^6	15	38 44 45 49	44·0	—	—
10^7	6	38 42 43	41·0	44 51	47·5
5×10^7	16	52 60 61			
	17	49 51 54 71	56·9	—	—

In certain eggs treated with nicotine, two bright regions appear at different points on the cortex (Pl. 12*c*). The times at which these two cortical changes appear strongly suggest that they are each caused by spermatozoa entering the egg at the points in question. The start of two cortical changes in the same egg has been observed and photographed five times, the second change appearing 6, 8·5, 9, 12 and 18 sec. after the first. This suggests that the block to polyspermy had not travelled completely round the egg within these times, and that it might therefore have the same velocity as the cortical change. On the other hand, if the block to polyspermy were a high-speed phenomenon, these effects could be explained if both spermatozoa activated the egg within a fraction of a second of each other, and if the latent period were different in the two cases.

CONCLUSION

The incidence of polyspermy when nicotine-treated eggs are inseminated at different sperm densities shows that this treatment increases the chance of an effective sperm-egg collision, by a factor which may be as much as 100, during the propagation of the block to polyspermy. This is not due to any effect of nicotine on the spermatozoa. If the observed cortical change is the block to polyspermy, the effect of nicotine is not to retard its conduction velocity but to increase the probability of a sperm-egg collision being successful. The experiments cannot be harmonized entirely with this hypothesis, or for that matter with the alternative hypothesis involving a high-speed block to polyspermy.

SUMMARY

1. Unfertilized eggs of *Psammechinus miliaris* which have been allowed to stand in sea water containing nicotine and are then inseminated in normal sea water are polyspermic.

2. Polyspermy is not due to an increase in the speeds at which spermatozoa swim in these circumstances; nor to a decrease in the rate at which the change in cortical structure of the egg, the first sign of fertilization, is propagated over the egg surface.

3. Experiments to test alternative explanations of the effect of nicotine have been carried out.

4. The experiments do not enable a firm decision to be made between (*a*) a high-speed block to polyspermy (which has not been observed) with a high probability of a successful collision, and (*b*) a low-speed (20 sec.) block to polyspermy with a low probability of a successful collision.

When discussing the morphology of inseminated oocytes in a previous paper (Rothschild & Swann, 1949), we referred to Seifriz's work on this subject (1926), but not to that of E. B. Harvey (1938) and in particular to her remarkable photographs of protoplasmic papillae on the surfaces of inseminated oocytes. We welcome the opportunity of rectifying this omission.

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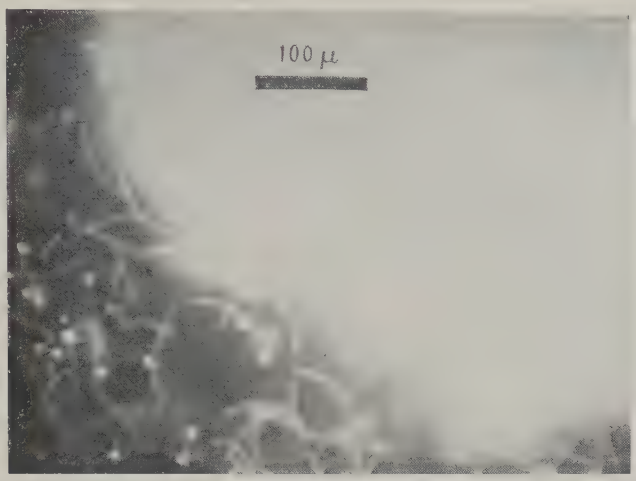
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EXPLANATION OF PLATE 12

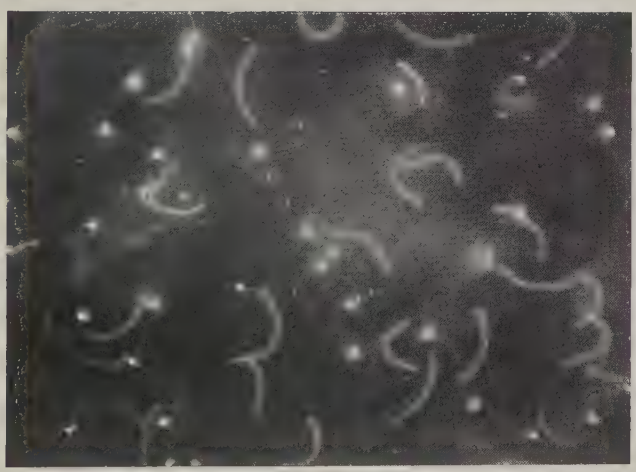
a. Tracks of sperm heads (*P. miliaris*) in the neighbourhood of undiluted semen, under dark ground illumination. Exposure, 0·5 sec. $T°$ C., 17.

b. Tracks of sperm heads (*P. miliaris*) in a suspension of density 10^5/ml., under dark ground illumination. Exposure, 0·5 sec. $T°$ C., 17. Magnification as in Pl. 12*a*.

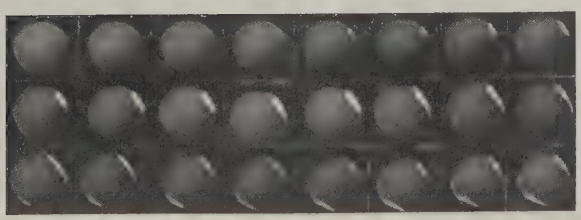
c. Propagated cortical changes in dispermic egg of *P. miliaris*, pre-treated with nicotine. Dark ground illumination. Exposure, 0·38 sec. Interval between photographs, 0·75 sec. $T°$ C., 17. Diameter of egg, 100μ.



a.



b.



c.

ROTHSCHILD AND SWANN—FERTILIZATION IN SEA-URCHIN EGG

THE DETERMINATION OF SODIUM IN SMALL VOLUMES OF FLUID BY FLAME PHOTOMETRY

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(*Received* 16 *May* 1950)

(With Two Text-figures)

INTRODUCTION

A method of measuring the freezing-point of small quantities (10^{-3} cu.mm.) of fluid has recently been described (Ramsay, 1949). This method opens up certain lines of investigation hitherto impracticable owing to the small quantities of fluid available. But measurements of total osmotic concentration as given by freezing-point are of limited value by themselves, and it is obviously useful to develop analytical methods which will give the concentrations of the various substances present in the fluid. The prime requirement is that such methods shall be applicable to samples of this same order of size; even fairly rough approximations might give valuable information in the present state of knowledge.

There are difficulties in reducing the scale of operation of ordinary chemical methods. These difficulties are first apparent in gravimetric work, then in volumetric and finally in colorimetric work, when quantities eventually become too small to give a measurable depth of colour. It is natural, therefore, that attention should turn to spectrometric methods which are less subject to these limitations, and more particularily so because the alkali metals sodium and potasssium, which are rather difficult to determine chemically, give intense and easily excitable emission spectra.

A general account of the use of spectrographic methods in biological problems has been given by Mitchell (1947, 1948). It is a common feature of such investigations that the element to be studied is present in very low concentration in a material which is available in more than adequate quantity. In the present problem we have a different situation, the element to be studied being a major constituent of the medium but only very small quantities of the medium being available.

In the first situation it is generally necessary to employ high-energy methods of excitation—arc or spark—and a spectrometer of high quality is required to separate the lines of the trace element from the lines produced by the other elements in the material. Conventionally, the spectrogram is recorded on a photographic plate and the intensities of the various lines are measured with a densitometer; even if the lines are weak the abundance of material makes it possible to give a sufficiently long exposure. The development of high-amplification photo-multiplier tubes has now made direct reading methods practicable. With stable conditions of excitation steady levels of intensity can be determined (Dieke & Crosswhite, 1945); alternatively,

integrating devices can be used over a fixed period of exposure. Elaborate integrating instruments have been developed by Saunderson, Caldecourt & Peterson (1945) and by Hasler, Lindhurst & Kemp (1948) for use in metallurgical processes.

In the second situation, where the amount of material may be so small that measurable intensity is sustained for only a fraction of a second, the value of integrative methods is obvious. The photographic method is, of course, integrative but infinitely more laborious than the photo-electric method. Another feature of the second situation is that the emission spectra of the alkali metals can be excited at the relatively low temperatures of the air-coalgas flame which are insufficient to excite most other elements, so that, even when emission of the alkaline earths calcium and magnesium have to be reckoned with, the flame spectrogram of a biological fluid remains relatively uncomplicated. It is therefore possible to separate the components of the spectrogram with a crude spectrometer or even with a system of filters. These considerations are, in fact, basic to the design of the instrument known as the flame photometer (for a recent review see Leyton, 1948).

A flame photometer is described by Barnes, Richardson, Berry & Hood (1945). The solution containing the element to be determined is atomized into a flame, and the light from the flame passes through an appropriate filter to a photo-cell and galvanometer. The application of this apparatus (and of the related Perkin-Elmer Model No. 18) to the determination of sodium and potassium in biological fluids is described by Hald (1947) and by Overman & Davis (1947). Other types of flame photometer are described by Belke & Dierkesmann (1948) and by Domingo & Klyne (1949). These authors were attracted to the method because of its speed for routine purposes, and were accustomed to use 0·1–2·0 ml. of blood, urine, etc., diluted as required, so that it is not easy from their work to discover the ability of these instruments to deal with small quantities. From the original description by Barnes we learn that 2–3 ml. of diluted fluid are required to obtain a reading. From Bills, McDonald, Niedermeier & Schwartz (1949) it appears that the Perkin-Elmer has a lowest standardization range of 0–1 p.p.m., implying that 1 p.p.m. can be set to give a deflexion of 100 scale divisions. 2 ml. of a 1 p.p.m. solution contain 2×10^{-3} mg. sodium; human serum contains about 3·15 mg./ml. so that the volume of serum required to give 2 ml. of 1 p.p.m. sodium is 0·635 cm.mm.—more than 600 times greater than the volume specified as a prime requirement. Existing flame photometers have two main disadvantages. First, only a portion of the sample is effectively atomized into the flame, the rest going to waste; secondly, a steady level of emission is required for a reading, and during the time taken for the instrument to settle down some of the sample is burned away to no purpose. These two disadvantages can be avoided by the use of a method whereby the whole of the sample is passed through the flame and the total emission is integrated. In addition, the use of photo-multiplier tubes offers possibilities of greater sensitivity.

When considering the possibility of applying integrative flame photometry to this problem of dealing with small volumes I was fortunate in meeting Dr T. M. Sugden who had already made use of the principle on a larger scale (Belcher & Sugden, 1950). I am glad to have this opportunity of thanking Dr Sugden for the information

and advice he has given me on various aspects of chemical spectroscopy. I was also fortunate in that this problem aroused the interest of Mr S. W. H. W. Falloon, and in collaboration with him and with Mr K. E. Machin the spectrometer and integrating system were designed and built. This apparatus is described in detail elsewhere (Ramsay, Falloon & Machin, 1951). The purpose of the present paper is to describe the techniques which are special to the use of this apparatus with small samples of biological fluid.

PRINCIPLE

A small volume—of the order of 10^{-3} cu.mm.—of fluid containing sodium is drawn up into a silica capillary pipette to a mark. This sample is then transferred to a platinum wire and dried. The platinum wire is thrust into an air-coalgas flame by a mechanical device. The light from the flame enters a spectrometer; the spectrum falls upon a mask having a slit which allows the sodium D lines to pass through to the cathode of a photo-multiplier tube. The photo-multiplier tube is connected to an integrating circuit and the reading is presented on a meter. The instrument is calibrated with known solutions of sodium which are drawn up to the same mark in the capillary pipette.

APPARATUS

This apparatus is fully described elsewhere (Ramsay, Falloon & Machin, 1951), and only a bare outline will be given here.

The platinum wire is carried into the flame by a rotating arm, actuated by a spring and set in motion by a cable release.

The burner is of the Meker type, using coalgas and compressed air; it is surrounded by an outer tube or sleeve which carries filtered air so that dust-laden air from the room is not carried into the flame. Owing to pressure variations in the air and gas mains it is necessary to use air and gas reservoirs of the gasometer type which can act as regulators or can be used as the sole source of supply just when the observation is to be made.

The spectrometer has a slit 1 in. in height and 0·015 in. in width. The lens system is of 2·9 in. aperture and a large carbon disulphide prism is used. The photo-multiplier, type 931 A, has a stabilized power supply. The output of the photo-multiplier is taken via a cathode follower to a Miller integrator. The meter effectively records the change in anode voltage of the integrator. It can be adjusted to zero before each reading. The meter scale is 0–250 and can be read to the nearest unit.

In order to cut down the amount of light when more concentrated solutions are being tested an iris diaphragm is mounted just in front of the slit.

MANIPULATION OF THE SAMPLE

The use of fine silica capillaries for manipulating small volumes of water under liquid paraffin is described under the freezing-point method (Ramsay, 1949), and the technique used in the present work follows the same lines. The pipette into which the sample is drawn resembles a 'freezing-pipette' in that it is pulled from 1 mm.

diameter thin-walled 'Vitreosil' silica tubing and has a long gentle taper. The bore over the terminal 5 mm. or so should be about 20 μ and as uniform as possible. This terminal portion is bent at an angle of about 30° to the rest. It is given a thin coat of Bakelite 'Damarda' lacquer which being hydrofuge serves to prevent water creeping along the outside. The lacquer also serves to hold a short (2–3 mm.) piece of glass rod about 20 μ in diameter against the capillary, so that the end of the rod can be used as a reference mark about 2 mm. from the tip (Fig. 1 A). The pipette is fixed with sealing wax to a mercury reservoir and screw-plunger, which in its turn is mounted on an adjustable stand and arranged so that the bent terminal portion of the pipette is horizontal. Mercury is driven to the tip, and is then withdrawn under liquid paraffin which is allowed to fill the tapering portion of the pipette.

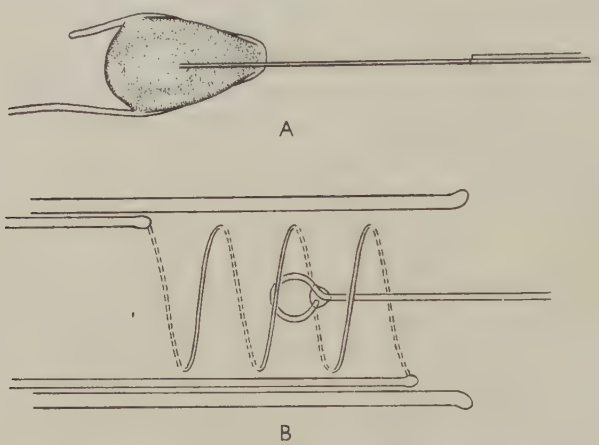


Fig. 1. A, the silica capillary pipette with tip inserted into drop of distilled water in platinum wire V; the sample about to be ejected. B, the platinum wire (which carries the sample into the flame) seen inserted into the tube containing the coil of nichrome wire. The drop from the storage tube is seen deposited upon the neck of the loop.

Subsequent operations are carried out under a medium-power binocular microscope. The droplet of the solution to be examined is kept under liquid paraffin in a watch-glass varnished internally with Bakelite lacquer. The watch-glass is placed on the stage of the microscope, the pipette is lowered into it, and by moving the microscope on the bench the droplet can be impaled upon the tip of the pipette. The solution is then drawn up into the pipette as far as the reference mark, the high viscosity of the liquid paraffin making control of this movement quite easy.

At this stage the sample might be transferred directly to the platinum wire which is to carry it into the flame, but since the process of drawing up and ejecting a measured sample takes time, and since the sensitivity of the apparatus is not constant over long periods, it is more convenient to accumulate several samples and to run them through the flame as rapidly as possible. The sample is therefore stored in another silica tube until required.

The storage tube is drawn in the same way as a 'freezing pipette', but is cut off so as to be about 100 μ in diameter at the tip and is lacquered and waxed on the

outside. It is mounted with sealing wax upon a short piece of ordinary glass tubing which pierces the cork of a small test-tube; the storage tube projects into the test-tube and is thus kept protected and in a moist atmosphere. The sample is transferred to the storage tube in the following way. A piece of thin (0·005 in.) platinum wire is mounted upon a base which slides over the stage of the microscope and carries the wire about 2 cm. above the stage. The end of the wire is bent into a V. This V is brought into the field of the microscope and a drop of distilled water, about 1 cu.mm., is deposited from a storage tube into the V. The pipette is raised, its tip is inserted into the drop and the sample is expelled—see Fig. 1 A. The drop now containing the sample is allowed to flow back into the storage tube.

The platinum wire which carries the sample into the flame is 0·015 in. in diameter, about 1·5 in. long and the free end is formed into a loop of about 0·1 in. diameter. This wire is permanently mounted on the arm which carries it into the flame. A low-power binocular microscope is arranged so as to be above the wire when it is in the 'out' position. The drop from the storage tube is blown out and deposited upon the neck of the loop. A coil of nichrome wire, formed inside a glass tube of about 0·4 in. internal diameter and heated to a dull red, is passed over the wire and held in position until the drop has evaporated (Fig. 1 B). The sample is then ready to be passed into the flame.

The meter and the various other controls are grouped at the photo-cell end of the spectometer. The air and gas valves in the 'off' position connect the mains to the gasometers, but the gasometers are not connected to the burner (except for a pilot flame). The valves are first moved to an intermediate position so that mains, gasometers and burner are interconnected and the flame is lit. The meter, which responds slightly to the lighting of the flame, is brought approximately to zero. The valves are then moved to the position where the burner is connected only to the gasometers and is isolated from the mains. The exact reading of the meter is noted and the cable release is pressed. The maximum excursion of the meter is read, corrected for the zero reading, and the net deflexion is recorded.

Given droplets of solution (uncontaminated with protein—see later) in watch-glasses under liquid paraffin, it is possible for a single operator to measure out and store twenty samples and then to run them through the flame in just under 2 hr.

ACCURACY

Before considering the accuracy of this method on the determination of sodium it is worth noting in passing certain results obtained while the apparatus was being developed—for details see Ramsay *et al.* (1951). With short flashes of light controlled by a shutter the consistency of the photocell and integrator, expressed in terms of standard deviation, was 1·8 % of the mean value. With moderately small volumes—*c.* 0·25 cu.mm.—of thallium solutions, delivered from a microburette, the standard deviation was 2 % of the mean value. *A priori*, it is unlikely that better results will be obtained with the sodium in biological fluids, of which much smaller volumes have to be accurately delivered.

In the tests with sodium the same silica pipette was used throughout. Its volume,

determined approximately from its dimensions as measured with an eyepiece micrometer, was $0{\cdot}7 \times 10^{-3}$ cu.mm. With a 0·1 % solution of NaCl, that is, with samples containing $2{\cdot}75 \times 10^{-7}$ mg. sodium, twelve consecutive readings gave an average deflexion of 110·6, with a standard deviation of 3·66, or 3·3 % of the mean. The same number of blank determinations, in which distilled water was drawn up into the silica pipette and all the other operations were the same, gave an average deflexion of 1·5 with a standard deviation of 1·44.

The relation between deflexion and concentration of NaCl was investigated over five different ranges of concentration. The results are set out in Table 1. For each

Table 1

	Concentration		Deflexions	Average deflexion
	% NaCl	Arbitrary units		
Series I. Iris aperture 1 in.; empirical factor 76·5	0·05	3·8	59, 66, 65, 65, 63	64
	0·10	7·65	120, 124, 128, 127, 125	125
	0·15	11·5	170, 181, 179, 181, 181	178
	0·20	15·3	226, 229, 236, 234, 227	230
Series II. Iris aperture $\frac{1}{4}$ in.; empirical factor 23·6	0·0	0	2, 1, 0	1
	0·10	2·35	41, 40, 41	41
	0·20	4·7	80, 81, 74	78
	0·292	6·85	116, 118, 115	116
	0·40	9·45	152, 155, 148	152
	0·50	11·8	179, 184, 171	178
Series III. Iris aperture $\frac{5}{8\cdot 2}$ in.; empirical factor 13·9	0·25	3·5	65, 64, 59, 61	62
	0·50	6·95	112, 113, 110, 114, 116	113
	0·73	10·15	161, 162, 165, 159	162
	1·00	13·9	220, 212, 222, 216, 211	216
Series IV. Iris aperture $\frac{5}{8\cdot 2}$ in.; empirical factor 8·0	0·50	4·0	68, 68, 68, 66, 67	67
	1·00	8·0	126, 133, 129, 131, 132	130
	1·50	12·0	182, 180, 188, 186, 184	184
	2·00	16·0	227, 230, 223, 236, 214	226
Series V. Iris aperture $\frac{7}{6\cdot 4}$ in.; empirical factor 3·7	2·00	7·4	122, 117, 131	123
	3·00	11·1	165, 159, 164	163
	4·00	14·8	211, 206, 201	206
	5 00	18·5	226, 226, 230	227
	6·00	22·2	239, 252, 256	249

series average deflexion was plotted against concentration and a curve was fitted by eye; at this stage it was obvious that the shape of the curve was very much the same in Series I–IV. Since another variable, the aperture of the iris diaphragm, was present, it was not to be expected that these curves could be superimposed as they stood; but by multiplying the concentration figures by certain factors (arrived at empirically for each series so as to give the best fit) the curves could be brought approximately into coincidence above an abscissa in which concentration was expressed in arbitrary units. The empirical factor for each series is given in Table 1. In Fig. 2 the average deflexions have been plotted against concentration in arbitrary units and for Series I–IV the relationship is reasonably represented by the curve (drawn by eye). The interpretation placed upon this relationship is that the energy falling on the cathode of the photo-cell is directly proportional to the amount of

sodium on the platinum wire and that the non-linearity arises in the electrical side of the apparatus. The results of Series V, also shown in Fig. 2, do not conform to this relationship, which is therefore not applicable to solutions more concentrated than 2 % NaCl, or, in more general terms, to quantities of sodium exceeding $5{\cdot}5 \times 10^{-6}$ mg.

From the data of Series I–IV it is possible to see how this method of determining sodium is likely to work in practice. We will assume that three measurements are to be made with a known solution in order to calibrate the apparatus for the conditions of the moment, and that it can then be used for determining sodium on unknown solutions for a limited period. An example will make this clear; the

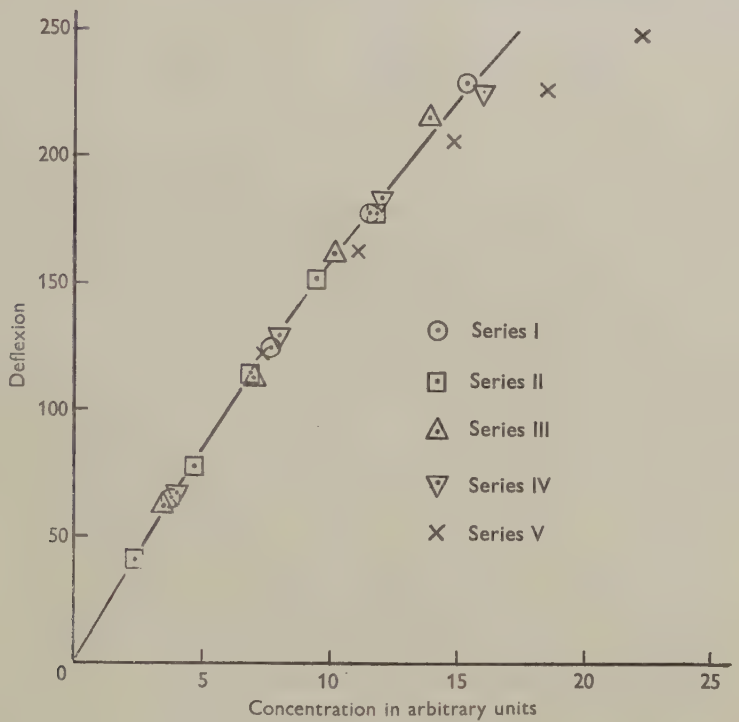


Fig. 2. For explanation see text.

treatment of Series I will be considered in detail—see Table 2. We take 0·1 % NaCl as the calibrating solution and imagine that the first three readings are our calibrating measurements. These three readings have an average value of 124. From the curve of Fig. 2 a deflexion of 124 corresponds to 7·55 arbitrary units of concentration; that is to say, for the conditions of the moment 7·55 arbitrary units can be equated to 0·1 % NaCl. We take first 0·05 % NaCl as an unknown solution. The deflexion given by each sample (column 1) is converted by means of the calibration curve into arbitrary units (column 2) and then converted into % NaCl by simple proportion from the relation established above. The error in concentration is given in column 4 and the percentage error in column 5. It is assumed that in practice the calibrating

solution will be chosen to give a deflexion of 100–150; in this particular analysis the following have been taken as the calibrating solutions:

Series I:	0·10 % NaCl	Series III:	0·73 % NaCl
Series II:	0·292 % NaCl	Series IV:	1·00 % NaCl

Table 2

(Series I: iris aperture 1 in.; calibrating solution 0·10 % NaCl; calibration readings 120, 124, 128, av. 124 ≡ 7·55 arbitrary units. 1 arbitrary unit ≡ 0·01325 % NaCl. 'Unknown', 0·05 % NaCl.)

Deflexion	Arbitrary units	% NaCl	Error	% error
59	3·45	0·0457	0·0043	−8·6
66	3·85	0·0509	0·0009	+1·8
65	3·8	0·0503	0·0003	+0·6
65	3·8	0·0503	0·0003	+0·6
63	3·75	0·0497	0·0003	−0·6

In all, the percentage errors of fifty-six samples have been calculated and they have an average value of 2·93 %. These errors are compounded of the systematic error of calibration and the random error of the individual sample and it is therefore difficult to express them in terms of standard deviation.

This treatment, whereby the accuracy of the method is assessed from data which have already been used in establishing the form of the calibration curve, is possibly open to objection on theoretical grounds. An alternative procedure, avoiding the use of a calibration curve, is to take a measurement on an unknown sample, to compare it with measurements on two known samples, one of higher and one of lower concentration, and to determine the unknown by linear interpolation. Taking Series II we can treat 0·10 % NaCl as unknown, and imagine that distilled water and 0·20 % NaCl are the calibrating solutions; similarily with 0·20, 0·292 and 0·40% , *mutatis mutandis*. This treatment provides the errors on twelve samples from Series II alone, and the average error is found to be 3·12 %. All in all, it is probably not wide of the mark to assess the average error of the method at about 3 %, for pure solutions of sodium chloride and for less than $5{\cdot}5 \times 10^{-6}$ mg. sodium. In practice, of course, we have to deal with solutions in which other substances are present and may give rise to error in other ways. These matters are discussed below.

SOURCES OF ERROR

Only a small fraction of the light energy emitted from the flame is made use of by the photo-cell; the success of any method of flame photometry depends in very great measure upon the possibility of controlling other variables so that the amount of light falling on the cell is related only to the amount of element introduced into the flame. Careful stabilization of gas and power supplies is an obvious precaution. The relative importance of other factors has not been fully investigated, but experience in the present work has shown that the thickness of the platinum wire, its height above the burner, the orientation of the loop and the disposition of the salt upon it are all highly significant. Consistency is achieved by strict attention to a rigid routine

in which the same procedure is followed in every detail. This is attended with good results when pure solutions of sodium chloride are used, but when biological fluids are used two other sources of error become apparent. The first is the effect of protein—and presumably of other surface-active substances—on the process of measuring out the sample in the silica pipette (volumetric error). The second is the effect of one element upon the emission of another (interference error).

(1) *Volumetric error.* The difference between the standard deviation of 2 % obtained with thallium delivered from a micrometer burette and the standard deviation of 3·3 % obtained with sodium delivered from a silica pipette as described may well be due to the error in measuring out the much smaller quantities of fluid. When one considers that relative to the volume of fluid the area of contact with the wall of the silica pipette is very large, it is perhaps surprising to find the error so small. No measurement of the contact angle between water, liquid paraffin and silica has been made, but inspection of the interface in the pipette shows that the water tends to displace the paraffin from the surface; yet results indicate that when the sample is ejected the amount of water remaining must be extremely small. A test was carried out in which the pipette was first washed out with 1 % NaCl and then two samples of distilled water were taken; if appreciable quantities of watery fluid adhere to the walls of the pipette the first sample of distilled water should give a higher deflexion than the second.

First sample	Second sample
6	2
2	2
3	0
2	2
Av. 3·25	Av. 1·5

The value of 1·5 for the second sample is identical with the value quoted in the previous section for twelve successive samples of distilled water; from the difference between 3·25 and 1·5 it can be calculated that all but 3 % of the sample is ejected.

Conditions are very different when the solutions used contain protein. When a solution containing protein is drawn into the pipette for the first time it appears to behave more or less normally, and if it is ejected slowly (the process taking about 30 sec.) the paraffin appears to replace it completely. But when the attempt is made to draw up a second sample the solution shows a strong tendency to creep rapidly up the walls of the pipette in an irregular manner, and generally fails to displace the paraffin completely. This trouble can be avoided by cleaning out the pipette after each protein-containing sample has been handled. It is sometimes necessary to resort to powerful cleaning agents such as dichromate-sulphuric acid, but since this attacks the lacquer covering of the pipette it has to be used with care; in most cases normal hydrochloric acid is effective, as is shown by the following test.

Pure lactoglobulin (6 mg.) were added to 39 mg. of distilled water (acidified with CO_2) in a silica tube and brought into solution. Three test solutions were prepared: (*a*) 2·0 % NaCl plus an equal volume of distilled water; (*b*) 2·0 % NaCl plus an

equal volume of the protein solution; (*c*) the protein solution alone. Four samples of each were taken, and after each sample had been ejected the pipette was washed out with normal hydrochloric acid. The deflexions were as follows:

(*a*) 155, 154, 155, 160, Av. 156
(*b*) 149, 175, 155, 152, Av. 157·75
(*c*) 1, 1, 3, 1, Av. 1·5

The presence of protein does not apparently introduce any important systematic error; it may possibly increase the random error, as in this case, but in other tests with protein-containing solutions this effect is not conspicuous.

(2) *Interference error.* Interference effects, in the broadest sense of the term, have been noted with all types of flame photometer. Some of the very large errors reported by Parks, Hilton & Lykker (1948), using the Perkin-Elmer no. 18, have been traced to the electrical circuits of the instrument itself by Bills *et al.* (1949) but even with improved design interference phenomena between different elements are recorded, in extreme cases giving errors of up to 50 %. Other cases of interference are due to imperfections of the filter system, e.g. the interference between sodium and calcium reported by Domingo & Klyne (1949). These authors also report that the interference between sodium and potassium found in the air-acetylene flame is not found in the air-butane flame. Results are also affected by the presence of organic substances such as sucrose and urea (Berry, Chappell & Barnes, 1946). Some of this interference may be indirectly the result of changes in the conditions of atomization—surface tension effects, for example; but there is also evidence that the interference is independent of the method used for introducing the material into the flame. The physical nature of interference is not fully understood.

A few tests have been carried out with the method under present discussion to discover the magnitude of the errors likely to affect the determination of sodium in the presence of some of the other constituents of biological fluids.

A sample of human serum known to contain 3·17 mg. sodium per ml.* was tested, using 1·0 % NaCl as calibrating solution; the results showed 3·34 mg. sodium per ml., an error of +5·5 %.

A sample of sea water known to contain 11·15 g. sodium per litre* was diluted with twice its volume of distilled water and the sodium was determined using 1·0 % NaCl as calibrating solution. The amount of sodium was found to be 10·29 g. per litre, an error of −7·7 %.

The effect of the sulphate anion was tested by comparing 1·0 % NaCl with 1·216 % Na_2SO_4 which has the same concentration of sodium. The apparent concentration of sodium in the Na_2SO_4 solution was lower by 6·75 %.

The separate effects of potassium, calcium and magnesium were studied in rather more detail in a series of tests, the results of which are given in Table 3.

An examination of this table and of the results of the other tests shows: (*a*) that the interference effects do not bear any simple relationship to the concentration of the interfering substance; and (*b*) that no error greater than 7·7 % has been recorded.

* I am indebted to Mr W. T. W. Potts for these analyses.

Table 3

(Concentration of NaCl 0·1 M throughout.)

Interfering Substance	Percentage error
0·01 M-KCl	+1·6
0·10 M-KCl	+6·9
0·90 M-KCl	+5·9
0·005 M-$CaCl_2$	−1·75
0·01 M-$CaCl_2$	−6·55
0·02 M-$CaCl_2$	−3·47
0·05 M-$CaCl_2$	0·0
0·10 M-$CaCl_2$	+1·64
0·90 M-$CaCl_2$	+4·37
0·005 M-$MgCl_2$	−1·1
0·01 M-$MgCl_2$	−1·68
0·05 M-$MgCl_2$	0·0

DISCUSSION

The possibilities of extending this method to deal simultaneously with other elements besides sodium have not yet been seriously considered. The potassium lines in the near infra-red are of the same order of intensity as the sodium D lines, but although photo-multiplier tubes covering the infra-red have been produced in America they are not on the market in Britain. It may be possible to use the weaker potassium lines in the violet with the 931 A which has a maximum sensitivity in this region. Calcium is rather less promising owing to the poor sensitivity of the 931 A to the red bands of calcium oxide. Flame photometry does not appear to have been applied as yet to the determination of magnesium.

As has already been mentioned, it is the saving of time in circumstances (e.g. in hospital laboratories) where a large number of routine analyses have to be carried out that has attracted attention to the possibilities of flame photometry. Unless steps are taken, as described below, to reduce the interference errors flame photometry is distinctly less accurate than orthodox chemical methods in which the nature and causes of error are more fully understood. But if it is a requirement that the method is to operate on 10^{-3} cu.mm. of fluid then no alternative to the spectrometric method appears to be available. Even the methods involving the use of radio-active isotopes have not yet been refined sufficiently to deal with these small quantities.

Evidence has been presented to show that the average error of a series of observations on the same fluid is about 3 %; for purposes of approximation we may assume that the error of any single observation is unlikely to exceed three times the average error, i.e. 9 %. The recorded systematic errors, due to interference, have not exceeded 7·7 %. This suggests that the method will give the sodium concentration in a single 10^{-3} cu.mm. sample of unknown composition to within 20 %. This is a large figure, but in view of the variation from one animal to another, especially among invertebrates even this would be useful accuracy. When something is known about the other substances present it should be possible to reduce this figure by about half.

Interference errors can be reduced in various ways. Belke & Dierkesmann (1948) first determine sodium, and then in the subsequent determination of potassium they use calibrating solutions of potassium chloride containing sodium chloride in appropriate concentration; similarly, the calibrating solutions for calcium contain sodium chloride and potassium chloride in previously determined concentrations. The error in the determination of sodium, potassium and calcium in blood, urine, etc., is thereby reduced to about 2 %, and in spite of the more complicated procedure the time saved is still substantial. This method, involving three successive measurements and therefore three samples, is not attractive when the quantity of solution is limited.

A more promising line of attack has been described by Berry *et al.* (1946). Their method depends upon the assumption (not unreasonable in view of their results) that interference effects upon related elements are similar. Thus, if a solution is to be analysed for sodium (or potassium) in the presence of interfering substances, an amount of another alkali metal, e.g. lithium, is added to bring the concentration of lithium in the sample to a known value. The lithium is then determined in the same way as the sodium and if the apparent concentration of lithium differs from the known concentration by a certain factor, that same factor is applied as a correction to the apparent concentration of sodium. This method of correction is of course inapplicable to cases of mutual interference by alkali metals (Bernstein, 1950). From the point of view of the manipulations involved the application of this principle to the method here under discussion does not appear to present any difficulty.

Of the possibility of reducing the volume of the sample below 10^{-3} cu.mm. it is not possible to say much. Naturally it will depend a great deal upon the concentration. In its present form the method has given a standard deviation of 3·3 % with samples of 0·1 % NaCl, of volume $0{\cdot}7 \times 10^{-3}$ cu.mm.; this could hardly be reproduced on a 0·01 % solution owing to the error in reading small deflexions of the meter. On the other hand, with concentrations greater than 1·0 % NaCl, requiring as they do some reduction in the aperture of the iris diaphragm, it is clearly possible to use smaller quantities of solution. The factors affecting the accuracy of measuring out the sample have received only a superficial examination so far. It may well prove possible to develop a better shape of pipette, with a smaller surface/volume ratio and with a local constriction at the reference mark. A substantial reduction in volume, say by a factor of 10, is possible but problematical.

SUMMARY

1. A method of integrative flame photometry is described, by which the amount of sodium in biological fluids can be determined, using samples of the order of 10^{-3} cu.mm.

2. With pure solutions of sodium chloride, of concentration from 0·05 to 2·0 %, the average error of the method is about 3 % of the concentration.

3. When other substances are present systematic errors of up to 7·7 % have been recorded.

4. The possibilities of reducing these systematic errors and of extending the method to deal with other elements are discussed.

I wish to thank Dr R. L. Mitchell for drawing my attention to certain papers on flame photometry which have appeared since his recent review.

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THE RESPIRATION OF SEA-URCHIN SPERMATOZOA

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(With Four Text-figures)

INTRODUCTION

Gray (1928) found that dilute suspensions of sea-urchin spermatozoa in sea water consumed more oxygen during their active life and respired at a higher rate, per unit quantity of spermatozoa, than dense suspensions. These phenomena are together known as the Dilution Effect. In this paper the first of these two characteristics is called Dilution Effect I, while the increased rate of O_2 uptake by dilute suspensions is called Dilution Effect II. The term Dilution Effect is sometimes used in the field of mammalian sperm physiology, with a different meaning; for in this case, it refers to the *decline* in the viability of sperm suspensions when diluted too much. In such circumstances mammalian spermatozoa may lose some substance to the suspending medium, with concomitant impairment of motility (Emmens & Swyer, 1948).

Such expressions as 'dense suspension' and 'dilute suspension' require precise definition. The most satisfactory way of expressing sperm concentrations is in terms of the number of spermatozoa per ml. of suspension. Other methods, involving measurements of the amount of nitrogen in the suspension, or the dry weight of the spermatozoa, are not so satisfactory, for reasons that have been discussed in a paper on counting spermatozoa (Rothschild, 1950*a*). Suggested definitions of 'very dense', 'dense', and 'dilute', are as follows (Rothschild & Tuft, 1950):

Very dense	$d \geqslant 5 \times 10^9$ sperm/ml.,	d = density.
Dense	$4 \times 10^8 < d \leqslant 10^9$ sperm/ml.	
Dilute	$d \leqslant 4 \times 10^8$ sperm/ml.	

These definitions apply to the semen of *Echinus esculentus*, in which the concentration of spermatozoa is about 2×10^{10}/ml. The three concentrations given above are therefore equivalent to the following dilutions of the original semen with sea water: 1 : 4, 1 : 50 to 1 : 20 and 1 : 50. The range of concentrations between 5×10^9 and 10^9 sperm/ml. was deliberately left undefined.

Dilution Effect II was observed between suspensions containing $8{\cdot}00 \times 10^8$ and $5{\cdot}58 \times 10^8$ sperm/ml. by Rothschild & Tuft (1950). Gray (1928), measuring sperm concentrations in terms of mg. N/ml. suspension, which have been converted to sperm/ml. on the basis of his figures and the average value of 2×10^{10} sperm/ml. in undiluted semen, observed Dilution Effect II in the following pairs of experiments (the figures are necessarily approximate):

6×10^9 and 3×10^9 sperm/ml.
4×10^9 and 2×10^9 sperm/ml.
2×10^9 and 1×10^9 sperm/ml.

In all these, the sperm suspensions were either 'very dense' or between 'dense' and 'very dense'. When a comparison was made between suspensions containing 8×10^8 and 4×10^8 sperm/ml. no Dilution Effect II was observed. Barron, Gasvoda & Flood (1949) also noted Dilution Effect II in the spermatozoa of *Arbacia punctulata*. They state that the maximum Q_{O_2}, 19·6, was attained in this species at a dilution of 1 : 200. These experiments were carried out on semen which was centrifuged for 10 min. at 2000 r.p.m., the supernatant seminal plasma being removed. After this, 'the remaining sperm was brought to the desired dilution starting with a stock dilution of 1 : 10 or 1 : 20' (p. 44). This probably means that a dilution of 1 : 10 in these conditions corresponds to a dilution of the original semen of 1 : 15 or 1 : 20. Apart from the difficulty in knowing the true sperm concentrations in these experiments, no details of temperature nor the method of effecting the dilution are given. This latter point is important, for if on dilution there is a sudden burst of respiration, dilution must be performed within the measuring apparatus, after temperature and gas equilibration. Otherwise the burst of respiration will be lost during the 15 min. or so needed for dilution and equilibration. The fact that it would be difficult if not impossible to achieve a dilution of 1 : 1600 within an ordinary Warburg manometer may explain why these workers found a Q_{O_2} of zero at this dilution. During the 15 min. before measurements could be begun, the suspension finished its O_2 consumption.

The Dilution Effect has been said to show first, that when dilute, sea-urchin spermatozoa have more room to move and therefore move more, consuming more O_2 in the process (see, for example, Barron *et al.* 1949). Secondly, that these spermatozoa do not die or senesce purely because of lack of fuel (endogenous substrate), but also because of some form of self-intoxication, using this word in a very general sense. There are certain difficulties in fitting these facts into a consistent picture of sea-urchin sperm metabolism, and it therefore seemed advisable to re-examine the basic features of their respiration under rigorous conditions. The experiments described in this paper were done with that object and, apart from clarifying some of the issues mentioned above, they may facilitate interpretation of some of the anomalous results obtained in the study of sea-urchin sperm metabolism, which have recently been tabulated in a review (Rothschild, 1951).

MATERIAL

Semen of *Echinus esculentus*. This consists of spermatozoa and seminal plasma. The latter is a viscous transparent fluid, sometimes yellowish and sometimes pinkish in colour. It contains fats, about four times as much potassium as sea water or coelomic fluid (Rothschild, 1948*a*), and in certain conditions, the substance Androgamone I, called A. I (Hartmann, Schartau, Kuhn & Wallenfels, 1939), which is not a protein. The semen was not washed before use for the following reasons. Washing consists in centrifuging the semen, removing the supernatant seminal plasma, and re-suspending the spermatozoa in sea water, the procedure usually being repeated three times. It damages or reduces the viability of the spermatozoa if done thoroughly (see later), while if not done thoroughly, as in the experiments of Barron *et al.* (1949),

when the semen was centrifuged for 10 min. at 2000 r.p.m. and not re-suspended until required for an experiment, there is little point in the procedure as the objective—to remove all the seminal plasma—is not achieved.

METHOD

Sperm counts. Photoelectric absorptiometer. This method of counting spermatozoa, which has been described in detail (Rothschild, 1950*a*), involves obtaining a relationship between the density of a suspension, determined with a haemocytometer, and the absorptiometer reading for the same suspension.

Oxygen uptake. The results described later are closely bound up with the question of adequate O_2 saturation of the sperm suspensions in the manometer vessels. The apparatus used for measuring O_2 uptake must therefore be described in some detail. Measurements were made with Warburg manometers, volume to manometer fluid approx. 15 ml., and differential manometers of about the same volume. The vessels were of conventional conical shape. They contained about 3, 2, 1·5 or 1 ml. of sperm suspension in different experiments. The shaking rate was 94 or 120 c.p.m., with a 4 cm. stroke in each case. The centre cup contained 0·1 ml. 10% KOH and the usual flowered filter-papers (Whatman no. 40) protruding above the top of the cup. The gas space contained air and the bath temperature was 15·0° C. The manometers were calibrated by the standard Hg method and also by liberating a known amount of N_2 in the flasks by the reaction $2KIO_3 + 3N_2H_4 \rightarrow 2KI + 3N_2 + 6H_2O$. The solubilities of N_2 and O_2 in water are sufficiently low and similar to make the error due to N_2 being liberated instead of O_2 negligible (Dixon, 1943).

RESULTS

Total O_2 uptake of dense and dilute suspensions, Dilution Effect I. Previous experiments in which Dilution Effect I was observed involved a comparison of the O_2 uptake of two suspensions, one containing about 3×10^9 sperm/ml. and the other 8×10^8 sperm/ml. One might suspect that O_2 saturation was a limiting factor in the O_2 uptake of the denser suspension, particularly as 6 ml. of suspension was used in the manometer flask. This was shown to be the case by the following experiment. A sample of semen containing $1{\cdot}78 \times 10^{10}$ sperm/ml. was diluted six times with sea water and different amounts, 3, 2, 1·5 and 1 ml. of this suspension placed in four Warburg manometers. The surface area exposed to the air in the manometer vessel increases as the amount of suspension is decreased, while the thickness of the suspension naturally decreases. If O_2 saturation is a limiting factor, we should expect that the O_2 uptake per unit quantity of spermatozoa would be highest in the vessel containing the smallest quantity of suspension. This method of testing the adequacy of O_2 saturation is more satisfactory than the conventional one of varying the shaking speed (although this was done as well), for two reasons. First, it appears to be more sensitive (Table 1); secondly, high shaking speeds may damage sea-urchin spermatozoa (Rothschild, 1948*b*). The results of this experiment are shown in Table 1. Table 1 shows that at this sperm concentration, 3×10^9/ml., O_2 saturation

Table 1. O_2 *saturation in suspensions of sea-urchin spermatozoa*

Vessel no.	V_1	V_2	V_3	V_4
Main vessel	3·0 ml. sperm suspension	2·0 ml. sperm suspension	1·5 ml. sperm suspension	1·0 ml. sperm suspension
k_{O_2}	1·29	1·30	1·35	1·44
Time (min.)	O_2 uptake in μl. $O_2/10^{10}$ spermatozoa. Initial dilution, 1 : 6. The suspension contained $2{\cdot}93 \times 10^9$ sperm/ml.			
0–20	48	54	64	89
94 c.p.m. 20–40	40	47	51	65
40–60	34	40	47	55
60–80	31	38	44	60
120 c.p.m. 80–100	27	34	42	52
100–120	24	32	36	48

Table 2. O_2 *uptake of sea-urchin spermatozoa in sea water*

Vessel no.	V_1 (in duplicate*)	V_2 (in duplicate*)	V_3	
Main vessel	1·92 ml. sea water	1·96 ml. sea water	1·94 ml. sea water	Shaker, 94 c.p.m. Concentration of spermatozoa in semen, $1{\cdot}85 \times 10^{10}$/ml. Dry weight of 1 ml. semen, 142 mg.
Side arm	0·08 ml. semen	0·04 ml. semen	0·06 ml. seminal plasma	
k_{O_2}	1·31	1·35	1·30	

* For microscopical examination of spermatozoa.

is a seriously limiting factor in the O_2 uptake of the suspension. This applies in all four cases as the O_2 uptake in vessel 4 increased when the shaking speed was increased. Although unimportant in this context, the explanation of the *lack* of increase in O_2 uptake in vessels 1–3 at the increased shaking speeds is probably that even in these conditions the 'internal resistance' of the larger volume of suspension prevented adequate mixing. In vessel 3, the decline in O_2 uptake may have been arrested at the higher shaking speed. Further experiments, similar to that described

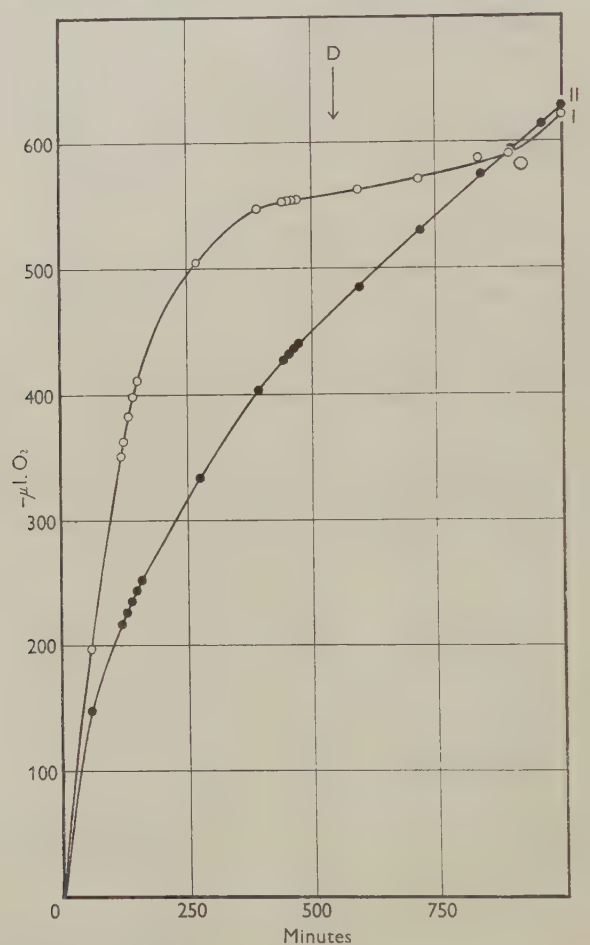


Fig. 1. Total O_2 consumed, per unit quantity of spermatozoa, by two sperm suspensions in sea water: I, sperm density, $7{\cdot}40 \times 10^8$/ml.; II, sperm density $3{\cdot}70 \times 10^8$/ml. Dilution of semen, I, 1 : 25; II, 1 : 50. D, 95 % of spermatozoa in more dilute suspension dead. O, increase in O_2 uptake due to bacteria and autolysis.

in Table 1, showed that the minimum permissible dilution, using 2 ml. of suspension, is 1 : 25, that is a sperm density of about 8×10^8/ml. In fact, it is safer to use concentrations between 1 : 30 and 1 : 50. However, to investigate Dilution Effect I, concentrations of 1 : 25 and 1 : 50 were used, to approximate as nearly as possible to the conditions in previous experiments. The details of an experiment on Dilution Effect I are shown in Table 2, and the results are shown in Fig. 1. The third

vessel, containing sea water and seminal plasma obtained by centrifuging semen for 10 min. at 2000 g. in an angle centrifuge, was a control to obviate the effects of O_2 uptake (if any) of seminal plasma, and to try and counteract the influence of bacterial metabolism towards the end of the experiment, which lasted some 18 hr. Seminal plasma had no O_2 uptake, though there was a small bacterial effect towards the end of the experiment; this has been appropriately subtracted from the values for sperm O_2 uptake obtained in the experimental vessels. At $t=0$, the contents of the side arms were transferred quantitatively into the sea water in the main vessels. In a normal manometric experiment, when a reagent is added from the side arms, the latter need only be washed into the fluid in the main vessel three times at the most. In this experiment, however, it was essential for all the semen, which is very viscous, to be mixed thoroughly with the sea water in the main vessel. To ensure this, the side arms were washed into the main vessels twenty times, which took about 2 min. The experiment shows that the total O_2 consumed, per unit quantity of sperm, by the denser suspension, so far from being less than that consumed by the dilute suspension, is in fact somewhat greater. There are some difficulties in determining the end-point because of autolytic and bacterial O_2 uptake after the spermatozoa have become motionless. These can be overcome in two ways. First, by simultaneously running two vessels whose contents and treatment are identical with those of the experimental vessels, from which samples are periodically taken for microscopic examination. By this method the time at which 95% of the spermatozoa in the dilute suspension were dead could be easily determined. It is possible to predict when this will occur, quite accurately, from examination of the O_2 uptake curve. Secondly, soon after the spermatozoa have become motionless, there is a marked and characteristic inflexion in the curve (Fig. 1, I), due to the onset of bacterial and autolytic O_2 uptake. Although all the spermatozoa in a suspension do not die at the same time, and the autolytic O_2 uptake of those which die early contributes to the 'true' O_2 uptake, the majority become motionless towards the end of the life of the suspension as a whole. This type of experiment was repeated at lower sperm densities, with the same result (Fig. 2). At such sperm concentrations Dilution Effect II is barely perceptible. The higher total O_2 uptake of the denser suspension is, however, visible.

The low rate of O_2 uptake of a 1 : 6 suspension and its lower total O_2 uptake are due to inadequate O_2 saturation, a high proportion of the spermatozoa in the suspension not respiring at all because of zero or nearly zero O_2 tension in perhaps two-thirds of the suspension. The experimental conditions are not entirely clear in the paper by Barron *et al.* (1949), but it seems probable that the low Q_{O_2}'s observed at high sperm densities, 1·7 and 3·6 at dilutions stated to be 1 : 10 and 1 : 30, had the same cause. There is of course no way of telling whether a biological tissue in a manometer is, or is not, suffering from inadequate O_2 saturation unless the shaking rate or the thickness of the material is varied, as the measured rate of O_2 uptake may satisfy the accepted requirements for the relationship between shaking rate and O_2 uptake, because O_2 saturation is inadequate.

These experiments show that Dilution Effect I, the increased total O_2 uptake of dilute suspensions, does not exist when the experimental conditions are physio-

logically satisfactory. When the conditions are not satisfactory the effect is due to spermatozoa suffering from anoxia.

Dilution Effect II. There are certain difficulties in the concept that the increased rate of O_2 uptake by dilute sperm suspensions is a result of the spermatozoa having more room to move in such suspensions. First, the energy expended by a spermatozoon is pre-supposed to be inversely proportional to the constraining forces of the system. Secondly, spermatozoa may move faster when diluted, but it does not follow that faster movement is a direct result of dilution. As chemical changes must precede the alterations in the structure of the sperm tail which produce the faster

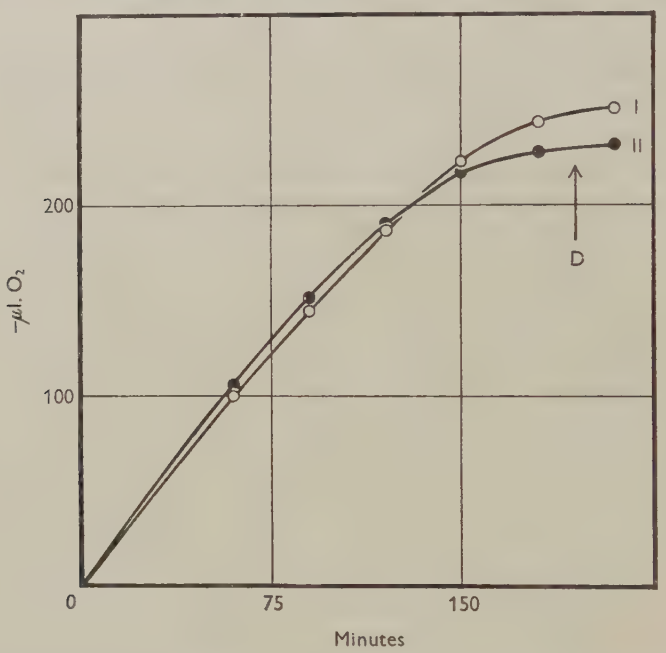


Fig. 2. Total O_2 consumed, per unit quantity of spermatozoa, by two sperm suspensions in sea water: I, sperm density, $4{\cdot}47 \times 10^8$/ml.; II, sperm density, $2{\cdot}24 \times 10^8$/ml. Dilution of semen, I, 1 : 50; II, 1 : 100. D, 95 % of spermatozoa dead.

movement, dilution may affect some aspect of metabolism first and this in turn may determine the subsequent movements of the spermatozoon. A different but plausible explanation is suggested by the concept, due to Hartmann *et al.* (1939),* that A.I, which inhibits motility, diffuses out of sea-urchin spermatozoa. They held that lack of motility in semen was caused by this substance. This is not true in the case of *E. esculentus* or *Psammechinus miliaris*, but is true in salmon semen (Runnström, Lindvall & Tiselius, 1944; Rothschild, 1951). If A.I were present in seminal plasma, the decrease in its concentration on dilution of a sperm suspension and consequent reduction of the inhibitory action of seminal plasma might explain the

* Southwick (1939) also claimed to have observed a sperm-paralysing substance in the seminal plasma of *Echinometra subangularis*. He did not consider the role of O_2 tension in inhibiting sperm movement in semen or seminal plasma, which makes his observations of questionable value.

increased O_2 uptake of dilute suspensions. This possibility is easily disposed of by the experiment shown in Table 3, in which a comparison was made between the respiration of spermatozoa in sea water containing different amounts of seminal plasma. High-speed centrifuges must be used to measure directly the ratio of seminal plasma to semen in a sample. As these were not available previously, the volume of a spermatozoon was estimated by graphical integration of oil-immersion phase-contrast photo-micrographs, and found to be 15–20 μ^3 (Rothschild, 1950*b*), the uncertainty being mainly due to difficulties in measuring the radius of the sperm tail. Through the kindness of Dr R. Markham, the Molteno Institute, Cambridge, the figure of 15–20 μ^3 has been compared with estimates based on centrifugation. A sample of semen was centrifuged for about 45 min. at 23,000 g. in an ultracentrifuge, the boundary between the spermatozoa and the seminal plasma being photographed when it had come to rest. The seminal plasma occupied about 47% of the volume of the sample which contained $2{\cdot}84 \times 10^{10}$ sperm/ml. A simple calculation shows that the volume of each spermatozoa is therefore about 18 μ^3. This will be discussed later in another context. In the sample in Table 3, the relative volumes of spermatozoa and seminal plasma per ml. semen were therefore about 0·35 ml. spermatozoa and 0·65 ml. seminal plasma. To bring the concentration of seminal plasma in a 1 : 50 sperm suspension up to the amount which a 1 : 25 suspension would contain, 0·013 ml. seminal plasma must be added per ml. sperm suspension. The experiment in Table 3 therefore shows that dilution of seminal plasma is not responsible for Dilution Effect II.

The next possibility to be investigated was that although no mutually inhibitory substances diffuse out of spermatozoa (Rothschild, 1948*b*), a substance might diffuse out of diluted spermatozoa, or off their surfaces, causing the affected spermatozoa to respire at a higher rate. Such an effect could be likened to the inactivation of a 'respiratory Pasteur enzyme' or the loss of some respiratory regulator. To test it, semen was diluted 1 : 50 with sea water and allowed to respire for 45 min. The suspension was then divided into four equal samples, 1, 2, 3 and 4. Sample 1 was centrifuged for 10 min. at 1100 g. and the spermatozoa were re-suspended in their own supernatant fluid, composed of sea water and seminal plasma. Sample 2 was centrifuged for 10 min. at 1100 g., after which the supernatant fluid was removed, sea water was added, up to the original volume, and the spermatozoa were re-suspended. Sample 3 was treated in exactly the same way as sample 2 except that after the supernatant fluid had been removed, half the quantity of sea water was added, so that after re-suspension the sperm density in sample 3 was double that in sample 2. Sample 4 was not centrifuged. Aliquots from all four samples were put in manometers and their O_2 uptake measured. In an experiment of this type, the decantation procedure cannot be assumed to achieve the desired result of doubling the sperm density, because variable numbers of spermatozoa are removed with the supernatant fluid. After the procedure outlined above, sperm counts were therefore made on the suspensions before they were put into the manometers. O_2 uptake was scaled according to these counts. The results of one of these experiments is shown in Table 4.

Table 3. *Effect of seminal plasma on O_2 uptake of sea-urchin spermatozoa*

Vessel no.	V_1	V_2	V_3	V_4	V_5
Main vessel	1 ml. sperm suspension* 0·1 ml. seminal plasma 0·9 ml. sea water	1 ml. sperm suspension* 0·05 ml. seminal plasma 0·95 ml. sea water	1 ml. sperm suspension* 0·025 ml. seminal plasma 0·975 ml. sea water	1 ml. sperm suspension* 0·013 ml. seminal plasma 0·987 ml. sea water	1 ml. sperm suspension — 1 ml. sea water
	$-\mu l.\ O_2$				
Time (min.) 0–40	50	54	54	52	55

* Semen, sperm density $1{\cdot}90 \times 10^{10}$/ml., diluted 1:25 with sea water. Shaker, 94 c.p.m.

Table 4. *Effect of centrifugation, removal of supernatant and artificial increase in sperm density on O_2 uptake of diluted sperm suspensions*

Vessel no.	V_1	V_2	V_3	V_4
Main vessel	2 ml. sample 1*	2 ml. sample 2*	2 ml. sample 3*	2 ml. sample 4*
	$-\mu l.\ O_2$			
Time (min.) 0–40	30	20	38	66
Spermatozoa/ml. suspension	$3{\cdot}51 \times 10^8$	$3{\cdot}44 \times 10^8$	$7{\cdot}11 \times 10^8$	$3{\cdot}51 \times 10^8$
$-\mu l.\ O_2$/40 min./10^9 spermatozoa	85	58	53	188

Shaker, 94 c.p.m. *, for contents of vessels, see text.

This experiment illustrates the following points:

(*a*) Centrifugation (c.f. V_1 and V_3, Table 4) markedly reduces the O_2 uptake of sea-urchin spermatozoa. This has been mentioned before (Rothschild & Tuft, 1950), but no quantitative information was given. Other workers, who have used washed sea-urchin spermatozoa in respirometric experiments, have not mentioned this fact.

(*b*) Seminal plasma has a protective effect on the spermatozoa (cf. V_1 and V_2 or V_3). This was known before (Hayashi, 1946; Rothschild, 1948*b*).

(*c*) There is no Dilution Effect II.

This experiment was repeated a number of times and the results are shown in Table 5. As the object was to confirm or reject the hypothesis that Dilution Effect II is caused by the diffusion of a regulatory substance out of spermatozoa in dilute suspensions, the controls, that is V_1 and V_4 in Table 4, were not always done and are

Table 5. O_2 *uptake of centrifuged sea-urchin spermatozoa, after respiring for* 45 *min.*

Exp. no. ...	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
$-\mu$l. O_2/40 min./10^9 sperm, h.c.*	27	28	31	33	32	39	37	29	31	18	20	35	36	27	28
$-\mu$l. O_2/40 min./10^9 sperm, l.c.*	29	27	27	29	35	22	22	25	23	24	25	23	26	30	36

* h.c., higher concentration; l.c., lower concentration. The figures have been scaled, after sperm counts, as in Table 4. See text for experimental details.

Table 6. O_2 *uptake of centrifuged sea-urchin spermatozoa, after respiring for* 10 *min.*

Exp. no. ...	1	2	3	4
$-\mu$l. O_2/40 min./10^9 sperm, h.c.*	19	23	23	19
$-\mu$l. O_2/40 min./10^9 sperm, l.c.*	45	32	29	47

* See footnote in Table 5.

omitted from the table. These experiments show that, *under these conditions*, there is a marked reduction in Dilution Effect II, which may mean that during the course of metabolism, some substance concerned with the regulation of respiration diffuses out of the spermatozoa. They do not, however, prove that this is the cause of Dilution Effect II and it is possible by a different experiment to show that another explanation must be sought. If the sperm suspensions are only allowed to respire for 10 min. before the centrifugation and concentration procedure, Dilution Effect II is much more pronounced (Table 6). This again suggests that 40 min. incubation in dilute suspension is associated with the loss of a regulatory substance from the spermatozoa but confirms that this is not the cause of Dilution Effect II. If the change on dilution were reversible, we should expect that after re-concentration in the way described above, there would be the same difference between dense and dilute suspensions, as regards O_2 uptake per unit quantity of spermatozoa, as there is before this treatment. Protective substances, such as albumin or seminal plasma,

probably act by reversing this effect or preventing its occurrence. The experimental conditions preclude the possibility that the dense and dilute suspensions were in different physiological conditions as a result of treatment, because both were treated in the same way until the moment of re-suspension. Though these experiments do not establish the cause of Dilution Effect II, they indicate that in other respects, the phenomena associated with the dilution of sea-urchin spermatozoa have much in common with those observed in mammalian spermatozoa, in which it is well known that in the absence of protective substances, dilution is followed by the loss of material from the spermatozoa and by impairment of viability.

If Dilution Effect II, instead of being thought of as the increased rate of O_2 uptake per unit quantity of spermatozoa in the more dilute suspension, is considered as the decreased rate in the more dense suspension, certain obvious questions come to mind. First, is some substance missing from the medium, or in short supply, with the result that the respiration of more dense suspensions is reduced? Secondly, can Dilution Effect II be artificially produced in suspensions which normally do not exhibit the effect, such as in the experiment shown in Fig. 2. Some preliminary investigations on the effect of copper and zinc on sea-urchin sperm respiration were recorded in a previous paper (Rothschild & Tuft, 1950). These experiments have been extended and it has been found possible to reverse Dilution Effect II by addition of $CuCl_2 2H_2O$ to the suspending medium. An example of an experiment in which this was done is shown in Fig. 3. In this experiment, which was done in duplicate, the main vessels of the manometers contained, (I) 2 ml. sea water; (II) 2 ml. sea water; and (III) 2 ml. sea water containing $CuCl_2 2H_2O$, final concentration 1·1 p.p.m. The side arms contained 0·2 ml. of semen, density $2{\cdot}35 \times 10^{10}$ sperm/ml., diluted 1 : 2·5 with sea water in side arms (II) and (III), and 0·2 ml. of the same semen, diluted 1 : 5 with sea water, in side arm (I). The slight initial dilution of the semen was to facilitate the quantitative transfer of the side arms to the main vessels, this being done by two people. The final sperm densities in the vessels were therefore: V_I, $4{\cdot}27 \times 10^8$; V_{II}, $8{\cdot}54 \times 10^8$; V_{III}, $8{\cdot}54 \times 10^8$. Fig. 3 shows that Dilution Effect II can be reversed by the addition of copper to sea water. There is a slight decline in the reversal during the second hour, which may be due to the precipitation of copper out of the sea water.

Dilution Effect II can be made to occur when the densities of the sperm suspensions are such that the effect is scarcely visible in the absence of special treatment. The reagent used to achieve this was sodium diethyldithiocarbamate (DDC), which inhibits reactions catalysed by Cu^{++} ions and copper-protein enzymes such as polyphenol oxidase. There are certain problems connected with its use (Keilin & Hartree, 1940, 1949), which are discussed later. One of these concerns the possibility that DDC exerts a narcotic action on the biological material; experiments have been done to investigate this possibility. Fig. 4 illustrates an experiment in which Dilution Effect II was imperceptible in two suspensions containing $8{\cdot}87 \times 10^8$ sperm/ml. sea water and $4{\cdot}44 \times 10^8$ sperm/ml. sea water (V_1 and V_2). When two identical suspensions, V_3 and V_4, were made up in sea water containing DDC, final concentration $3{\cdot}64 \times 10^{-5}$ M, the reduction in O_2 uptake of the denser suspension

was 62% while the reduction in O_2 uptake of the more dilute suspension was only 36%, in both cases after 90 min. More interesting still is the fact that in the untreated suspensions, V_1 and V_2, there was no Dilution Effect II, whereas in the treated suspensions, V_3 and V_4, there was a difference of 39% between the more dense and dilute suspensions after 90 min. respiration. If the inhibition of O_2 uptake were due to a narcotic action of DDC, or to the inhibitory effect of tetraethyldithiocarbamyl disulphide, a cytochrome *c*-catalysed oxidation product of DDC (Keilin & Hartree, 1940), one would not expect the percentage inhibition to vary so

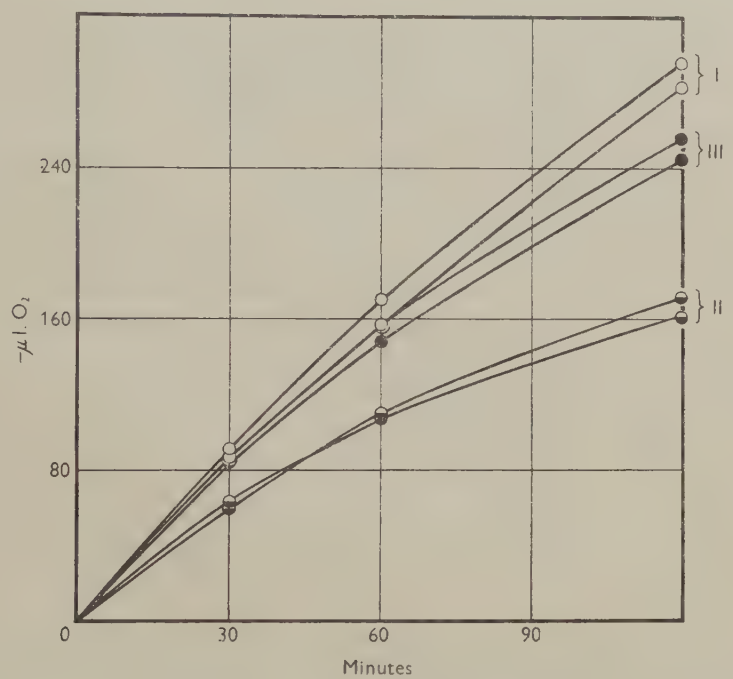


Fig. 3. Reversal of Dilution Effect II by copper. I, sperm density, $8{\cdot}54 \times 10^8$/ml., no copper; III, sperm density, $8{\cdot}54 \times 10^8$/ml., final concentration of $CuCl_2 2H_2O$, 1 p.p.m. II, sperm density, $4{\cdot}27 \times 10^8$/ml., no copper.

greatly according to the density of the suspension. The possibility that DDC does not act as a chelating agent, but as a narcotic, or in the way discovered by Keilin & Hartree, was investigated in another way, by varying the DDC concentration in the medium. The results of these experiments are shown in Table 7, which show without reasonable doubt that in these experiments DDC was acting as a chelating agent. There is, in fact, a suggestion that at the higher concentrations, DDC may have been utilized as a substrate by the spermatozoa, apart from exerting an inhibition.

Concentrations of $CuCl_2 2H_2O$. Previously (Rothschild & Tuft, 1950), the difficulties in knowing the true concentrations of copper salts in sea water were emphasized. The values given were said to be almost certainly too high. The same applies in this paper though it has been found that if the solutions are made up

immediately before the experiment, lower concentrations have the same stimulating effect on sperm O_2 uptake as higher ones made up some time before the experiment.

DISCUSSION

The experiments described in this paper enable considerable simplifications to be made in a description of sea-urchin sperm respiration and senescence. Furthermore,

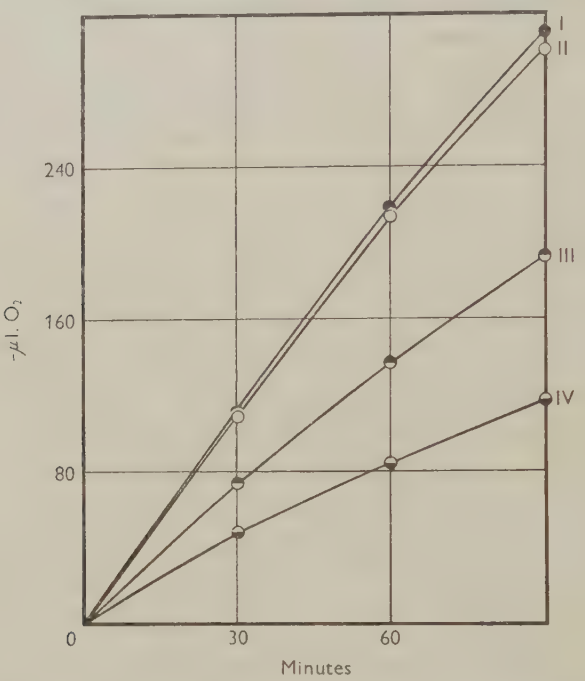


Fig. 4. Production of Dilution Effect II by sodium diethyldithiocarbamate (DDC). I, sperm density, $8{\cdot}87 \times 10^8$/ml., no DDC; II, sperm density, $4{\cdot}44 \times 10^8$/ml., no DDC; III, sperm density, $4{\cdot}44 \times 10^8$/ml., final concentration of DDC, $3{\cdot}64 \times 10^{-5}$ M; IV, sperm density, $8{\cdot}87 \times 10^8$/ml., final concentration of DDC, $3{\cdot}64 \times 10^{-5}$ M.

Table 7. *Effect of different concentrations of sodium diethyldithiocarbamate on the O_2 uptake of sea-urchin spermatozoa*

Molar concentration of DDC in sea water	$9{\cdot}71 \times 10^{-4}$		$9{\cdot}3 \times 10^{-4}$		$9{\cdot}3 \times 10^{-5}$		$3{\cdot}64 \times 10^{-5}$	
No. of sperm/ml., $\div 10^8$	9·46	4·73	8·67	4·54	8·87	4·44	8·87	4·44
Percentage inhibition of O_2 uptake	29	13	21	6	42	28	62	36

it now appears that the main difference between them and mammalian spermatozoa is that the former are almost entirely aerobic, while the latter, by virtue of their ability to break down fructose into lactic acid (Mann, 1946), are viable and can move in the absence of O_2. Both are adversely affected by too great dilution unless

protective substances are added to the diluent. There is now no evidence to support the view that sea-urchin spermatozoa in relatively dense suspensions suffer from some form of auto-intoxication, either of an intracellular or extracellular nature. Movement stops when the endogenous substrate or some essential link in the metabolic processes is exhausted. The view that the total O_2 uptake, per unit quantity of spermatozoa, is higher in dilute than in dense suspensions, must be revised provided oxygen saturation is adequate in both cases. Because of their high Q_{O_2}, which may be more than 30 at 15° C., inadequate oxygen saturation is a danger in all manometric experiments involving sea-urchin spermatozoa, unless the usual tests are made, or sperm counts show that the sperm density is less than 10^9/ml. For example, in a recent and interesting paper on the effects of egg jelly and Ca^{++} ions on the O_2 uptake of sea-urchin spermatozoa, Vasseur (1950, p. 393) used what he calls 'concentrated "dry" sperm... collected in the usual way by filtering through bolting silk', diluted 1 : 6 with sea water, in conical Warburg manometer vessels. The volumes of the vessels are not given. The semen of *Echinus esculentus* and *Psammechinus miliaris* was used. In the case of the former a semen dilution of 1 : 6 corresponds to a sperm density of about $3{\cdot}3 \times 10^9$/ml. Leaving aside the question of copper or zinc effects, this sperm density is undoubtedly too high for adequate oxygen saturation with 2·4 ml. suspension in the manometer vessels. This is confirmed by the increase in O_2 uptake when sea water was added to the suspensions as a control. The semen of *P. miliaris* has a higher sperm density than that of *E. esculentus*. Inadequate O_2 saturation cannot of course account for all of the increase in O_2 uptake Vasseur observed on addition of egg jelly and Ca^{++} ions. From what has been said above, the importance of making sperm counts is obvious.

The experiments involving the addition of $CuCl_2 2H_2O$ and DDC to sperm suspensions do not perhaps prove that the increased rate of O_2 uptake, per unit quantity of spermatozoa, by dilute suspensions, or more accurately, the decreased rate of O_2 uptake by dense suspensions, is due to the quantities of copper or zinc in sea water being marginal for these concentrations of spermatozoa. The experiments are consistent with this explanation, while for reasons that have already been given (p. 426), a metabolic interpretation of Dilution Effect II is needed. In undiluted semen, however, close packing undoubtedly interferes with sperm movement, when this is induced by an increase in O_2 tension. The O_2 uptake cannot be measured manometrically in these circumstances, though it might be possible to make measurements with an oxygen electrode. A quantitative examination of the possibility that the concentration of copper in sea water is marginal for dense suspensions has recently been made by Barnes & Rothschild (1950). However, the question probably does not arise in nature as the density of spermatozoa in the neighbourhood of a spawning sea-urchin is unlikely to be greater than 5×10^8/ml. and usually must be much less.

Considered by itself, the stimulating action of $CuCl_2 2H_2O$ might be held to lend support to a hypothesis, put forward by Barron, Nelson & Ardao (1948), to the effect that sea-urchin spermatozoa contain two kinds of —SH groups, soluble ones which regulate respiration, and fixed ones, present in the protein moiety of enzymes.

Low concentrations of —SH reagents are presumed to combine only with the first kind, causing an increase in respiration; higher concentrations combine with fixed —SH groups, with consequent inhibition of respiration. It is not obvious how the inhibition of O_2 uptake by diethyldithiocarbamate can be fitted into this picture. Moreover, in expounding this hypothesis Barron *et al.* make no reference to Dilution Effect II, the important feature of which is that the respiration of sea-urchin spermatozoa varies according to the density of the suspension. In another paper, however, on the effect of nitrogen mustards on sea-urchin spermatozoa (Barron, Seegmiller, Mendes & Narahara, 1948, p. 270), it is stated that 'the results seem to depend or vary with the concentration of sperm'. Apart from necessitating further work, the resolution of these differences in interpretation depends on future experiments being carried out under clearly defined conditions, an indispensable prerequisite of which is a knowledge of the number of spermatozoa in the experimental suspension.

In conclusion, if the 'trace metal'* explanation of Dilution Effect II is accepted, another distinction between mammalian and sea-urchin spermatozoa becomes resolved, for one difference between dilute and dense suspensions of sea-urchin spermatozoa is due to the medium and not the spermatozoa. Barron *et al.* (1949) found that the O_2 uptake of sea-urchin spermatozoa was markedly lower in artificial than in natural sea water, which may have the same explanation. In his original paper on this subject, Gray (1928) observed that other cases are known in which the biological activity of cells or organisms is increased by a diminution of population density. In some of these, competition for trace metals in the medium is a possible explanation which might be worth exploring.

As this is the first time that the total O_2 consumption of a known number of sea-urchin spermatozoa has been determined, it is of interest to relate this figure to the breakdown of endogenous substrate, assumed, purely for the purpose of the argument, to be 'glucose'. In the experiment illustrated in Fig. 1, $7{\cdot}40 \times 10^8$ spermatozoa (2 ml. of a suspension containing $3{\cdot}70 \times 10^8$ sperm/ml.) consumed 550 μl. O_2 in 400 min. If we further assume that the average O_2 uptake of each spermatozoon was $550 \div (7{\cdot}40 \times 10^8)$ μl. O_2, each spermatozoon must break down about $10^{-6}\mu$g. of 'glucose' in 400 min. This follows from the fact that the breakdown of 1 mole (180 g.) of 'glucose' is associated with the disappearance of $22{\cdot}4 \times 6$ l. of O_2. The volume of the middle piece of a spermatozoon of *E. esculentus* is not more than $5\mu^3$. If the whole of the middle piece contained 'glucose' solution, this solution would have to be about 20% to provide the necessary substrate. This concentration is rather high and suggests that the substrate may not be a carbohydrate and therefore that the respiratory quotient may be lower than one.

* The stimulating effect of zinc on sea-urchin sperm respiration (Rothschild & Tuft, 1950) was not investigated this year. According to Albert & Gledhill (1947) diethyldithiocarbamate chelates zinc and copper to the same extent. But if DDC reacts with a metallo-protein in these experiments, it should be remembered that this reagent has no inhibitory action on carbonic anhydrase.

SUMMARY

1. The O_2 uptake of sea-urchin spermatozoa, *Echinus esculentus*, has been re-examined under varying conditions of sperm density, and in the presence of $CuCl_2 2H_2O$ and sodium diethyldithiocarbamate (DDC).

2. The total O_2 uptake of dilute sperm suspensions was previously thought to be higher than that of dense suspensions per unit quantity of spermatozoa (Dilution Effect I). This result is only obtained when the oxygen saturation of the dense suspension is inadequate, which may easily occur as the Q_{O_2} of sea-urchin spermatozoa may reach 30 at 15·0° C. When oxygen saturation is satisfactory, the total O_2 uptake of dense solutions is slightly greater than that of dilute ones. The experiment cannot be done in micro-respirometers of the normal Warburg type unless the density of spermatozoa per ml. suspension is less than about 10^9, corresponding to an initial semen dilution of 1 : 20 or 1 : 25. These figures apply to other manometric experiments on the O_2 uptake of sea-urchin spermatozoa using normal amounts of material.

3. When movement ceases, there is a sharp increase in the O_2 uptake of the suspension.

4. The addition of seminal plasma to dilute sperm suspensions does not inhibit the increased rate of O_2 uptake, per unit quantity of spermatozoa, observed in these suspensions when compared with dense ones (Dilution Effect II). Dilution Effect II is therefore not caused by the dilution of an inhibitory substance in seminal plasma.

5. Sperm suspensions were prepared by diluting semen 1 : 50 with sea water and allowing them to respire for 45 min. They were then centrifuged, the supernatant was discarded and the spermatozoa were re-suspended to different densities with sea water. This treatment has the following effects:

(*a*) Centrifugation irreversibly damages the spermatozoa and reduces their O_2 uptake.

(*b*) Removal of the supernatant, which contains seminal plasma, and re-suspension in sea water also reduces O_2 uptake.

(*c*) The treatment markedly reduces Dilution Effect II.

If the experiment is done in the same way but the suspensions are only allowed to respire for 10 min. before centrifugation, (*a*) and (*b*) are the same, but Dilution Effect II is normal. This shows that during metabolism, a regulatory substance is lost from dilute suspensions, as in mammalian spermatozoa; but this is not the cause of Dilution Effect II.

6. Dilution Effect II, considered as the reduced O_2 uptake of dense suspensions, can be reversed by the addition of $CuCl_2 2H_2O$, 1 p.p.m., to the medium.

7. Dilution Effect II can be made to occur in sperm suspensions which do not normally exhibit it, by the addition of DDC, in concentrations as low as $3{\cdot}64 \times 10^{-5}$ M (final concentration). The action of DDC is not greater when its concentration is increased to 10^{-3} M, which suggests that in these conditions it acts as a chelating agent and not as a narcotic. For the same reasons its oxidation product, tetraethyldithiocarbamyl disulphide, is unlikely to be responsible for DDC's inhibitory effect on sperm O_2 uptake.

8. These results are consistent with the hypothesis that Dilution Effect II is due to the amounts of copper (or possibly zinc) in sea water being inadequate to satisfy the requirements of dense sea-urchin sperm suspensions. This situation is unlikely to arise during natural spawning as sperm densities are too low for the effect to occur in these conditions. Other interpretations of the stimulating action of copper and zinc are discussed.

9. The experiments remove several of the differences hitherto believed to exist between sea-urchin and mammalian spermatozoa.

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THE HATCHING MECHANISM OF MUSCID EGGS (DIPTERA)

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(With Three Text-figures)

INTRODUCTION

It is well known that the hatching of eggs in many insects, particularly Muscid flies, is markedly affected by humidity. This is well illustrated by the observation of Davies & Hobson (1935) that *Lucilia sericata* (Mg.) eggs which had been incubated under moist conditions and normally would have hatched in a few minutes, completely failed to do so when transferred to an atmosphere of 50% R.H. It has been assumed that delay or prevention of hatching by low humidity is largely a result of the chorion hardening under dry conditions.

The following observations are intended to throw light on the precise mode of action of humidity on hatching in the eggs of Muscid flies. In the course of the work, eggs of the following species were used, and the observations apply to them all: *L. sericata* (Mg.), *L. caesar* (L.), *L. illustris* (Mg.), *L. ampullacea* (Vill.), *Protophormia terra-novae* (R.–D.), *Calliphora erythrocephala* (Mg.) and *C. vomitoria* (L.).

HUMIDITY-DEPENDENT SHAPE CHANGES IN EGGS

Egg measurements were made by means of a microscope with ×6 eye-piece and 16 mm. (×10) objective and fitted with an eye-piece micrometer scale, so that the total magnification was ×60 (1 micrometer division = 16·47 μ). The above apparatus was found to permit fairly accurate measurements of the length changes of eggs, with a range or error of about ±6 μ. For the observation of width changes and the dimensions of certain smaller topographical features of Muscid eggs, attempts were made to use a higher magnification (×400). The low accuracy of these latter measurements do not permit a numerical evaluation of them but this limitation in the observations does not invalidate the hypothesis put forward in the following pages, linking these egg-shape changes with the hatching process.

Solid watch-glasses, sealed with flat plates vaselined at the edges, were used as humidity chambers, humidities being controlled within them by sulphuric acid/water mixtures (Wilson, 1921), 100% R.H. being maintained with distilled water. The cavity of each watch-glass was filled with the appropriate liquid to within 5 mm. of the underside of the lid. Eggs were attached to the underside of the cover plates, so that they lay within 5 mm. of the surface of the acid, and their dimensions could then be measured through the lid when the whole watch-glass was placed on the

mechanical stage of the microscope. Care was taken to use uniform-sized watch-glasses and similar thickness cover-glasses in all observations. Successive measurements on several eggs at various humidities were made by quickly transferring the cover-glass with its attached eggs on to another watch-glass containing the appropriate humidity solution, repeating the transfer for each R.H. All vessels were kept covered and airtight except during the short period of a few seconds when a transfer was being made. A period of 5 min. was allowed to elapse after eggs had been transferred to a new humidity, before measurements were taken, in order to allow the egg shells to reach equilibrium with the air of the chamber, and for the correct R.H. to be restored after the disturbance. Eggs were found to have different lengths at different humidities (see below). Successive length measurements made on eggs, from immediately after they had been transferred to a new humidity, until 30 min. later, showed that they reached their final length within 3 min. of the transfer. The length changes during humidity changes are thus seen to be very rapid.

Before describing in detail the shape changes observed, the external morphology of the Muscid egg must briefly be described. The eggs are roughly sausage-shaped, about five times as long as wide, the anterior end being occupied by a flatish circular area with the micropyle in its centre, and termed in this paper 'the micropylar plate'. On the dorsal surface of the egg, the chorion bears a pair of roughly parallel pleats (termed the 'hatching pleats' hereafter) running posteriorly from the edge of the micropylar plate for about four-fifths of the egg length. The area of the chorion between the hatching pleats is thickened (Davies, 1948) and is termed the 'hatching-strip' in the following account.

The shape changes observed may be summarized as follows:

(*a*) With increasing R.H. from 0 to 100% the eggs of all species gradually increased in length. Length measurements of eggs of three species at different humidities are given in Fig. 1. Differences in the amount of length change in the three species will be noted. The variation between individual eggs of one species in the percentage increase of length at 100% R.H. over the length at 0% (ten eggs in each case) is shown in Table 1. The points at each humidity in Fig. 1 show the amount of error

Table 1. *Variation in egg length with changes in humidity*

Species	Extremes of length at 0% R.H.	Extremes of length at 100% R.H.	Percentage range of variation in elongation
Lucilia sericata	1201–1272	1260–1338	4·6–7·3
L. caesar	1121–1203	1148–1232	3·2–6·3
L. illustris	1240–1310	1288–1370	3·8–6·3
L. ampullacea	1243–1342	1295–1379	4·2–6·8
Protophormia terra-novae	1150–1224	1226–1303	4·5–6·6
Calliphora erythrocephala	1452–1503	1482–1531	1·0–2·6
C. vomitoria	1186–1252	1210–1272	1·0–2·4

to be expected when measuring a distance of about 1200 μ in units of 16·5 μ. When eggs were transferred through the humidity series 0–100% R.H. several times upwards and downwards, the length values obtained at each humidity always coincided to within 4 μ.

(*b*) Accompanying the elongation described above, a reduction in the cross-sectional area of eggs occurred. The amount of this reduction was too small to be accurately measured with a magnification of $\times 60$. All that can be said is that it appeared to involve a reduction in the diameter of eggs of the order of 5%. A feature of this reduction in cross-sectional area was observable qualitatively. It occurred mainly by an inward movement of the dorsal side of the egg, the hatching pleat area. It is likely that the cross-section remained approximately circular during the shape changes (Fig. 2B).

(*c*) Simultaneously with the above shape changes, and probably forming an integral part of them, the hatching strip appeared to narrow and elongate slightly. Even with $400\times$ magnification, the hatching strip narrowing was too small to be accurately measured, since the distance involved is about 50 μ.

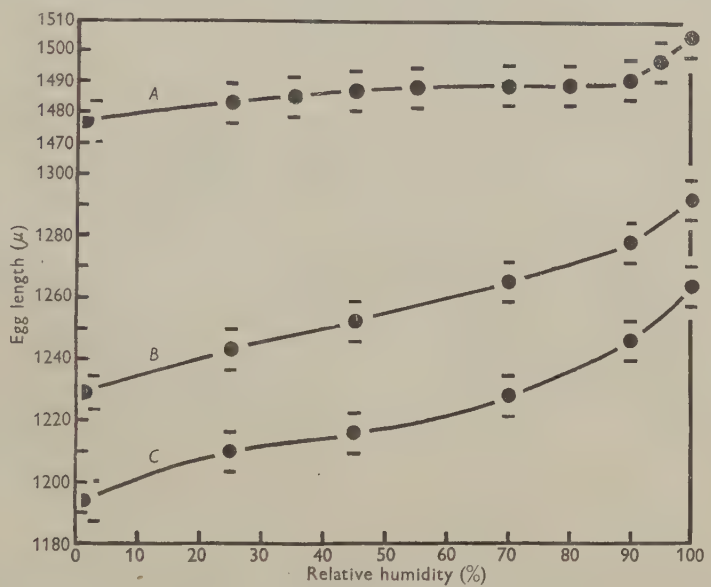


Fig. 1. Variation of egg length with humidity. Each curve is the average of 5 eggs. A, *Calliphora erythrocephala*; B, *Lucilia sericata*; C, *Protophormia terra-novae.*

These shape changes are represented diagrammatically in Fig. 2, and were further investigated, the results being given below as numbered observations for the sake of clarity:

Observation 1. When eggs were subjected to large humidity changes by transferring them from 0 to 100% R.H. directly, a proportion of the chorions split by a longitudinal fissure running closely along the outer margin of either of the hatching pleats. This fissure did not originate at any fixed point along the length of the pleats. Eggs which did not split during the first 0–100% R.H. change could be induced to do so by repeating the process 2–5 times. This shows that sudden large humidity changes caused strains in the chorion, making it split along one or other of two fairly constant lines. Only eggs in an early stage of development were used, so that splitting was

not due to larval activity within the eggs. With smaller humidity changes of 50–100% R.H. or 0–50% R.H., no rupture of chorions occurred, even after ten repeated changes. Thus with smaller changes in R.H., the changes in length were also smaller and the strains set up not sufficient to rupture the chorion.

Observation 2. The accessory gland secretions covering the outside of the chorion of the laid egg were removed by washing eggs in 1% sodium sulphide solution for 1 hr. Such eggs underwent shape changes in the same way and to the same extent as eggs with the secretion covering intact. This shows that the latter played no part in governing the shape changes.

Observation 3. Chorions were removed from eggs (cf. Evans, 1934) which retained their general shape since they were still enclosed by the intact vitelline membrane underlying the chorion. Such chorion-less eggs when subjected to successive

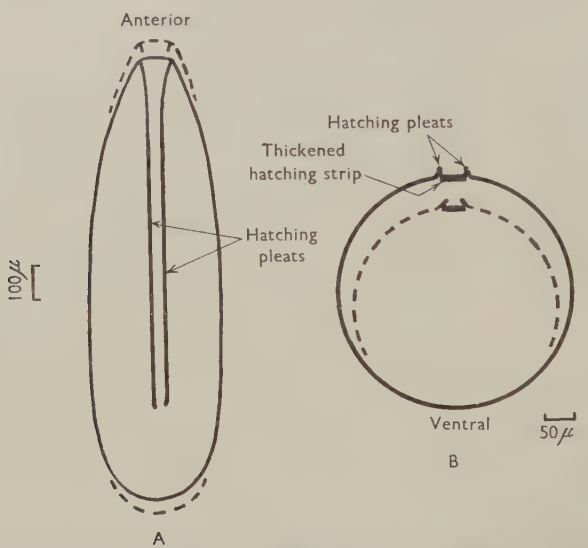


Fig. 2. A, dorsal view of egg showing length increase only; B, cross-section of egg showing reduction in diameter by inward movement of dorsal side. The amount of the diameter reduction is greatly exaggerated. ——, outline at 0% R.H.; – – – –, outline at 100% R.H.

humidities from 0 to 100% R.H. and vice versa, underwent no detectable shape changes. It is concluded, therefore, that the chorion alone was responsible for the humidity-dependent shape changes. The tendency of the Muscid chorion to alter shape at different humidities would be resisted by the egg it invests, thus causing strains to be set up in the former. The amount of strain will be humidity dependent because the shape of the chorion is humidity dependent.

Observation 4. That the shape changes caused by the chorion were not due to the effects of humidity on a small specialized area of the shell, such as the hatching strip and pleats, was demonstrated in the following way. The lengths of ten eggs at 0 and 100% R.H. were measured. The hatching strip and pleats of six of these eggs were carefully covered with waterproof cellulose paint. On the other four eggs a similar longitudinal strip of chorion was painted, but this time on the ventral side of the

egg, on unspecialized chorion. Only in the latter four eggs would the hatching strip be exposed to humidity changes. The lengths of the eggs at 0 and 100% R.H. were re-measured. It will be seen from the results in Table 2 that both groups of eggs elongated with rise in humidity, but to a smaller extent than before treatment, there being no significant difference between the two groups. The smaller length increase in both groups after treatment was probably due to the stiffening effect of the paint itself. It is considered therefore that the shape changes were not due to the properties of the hatching pleat region alone, but rather to the properties of the chorion as a whole.

Table 2. *Percentage increase in length of eggs from* 0 *to* 100% R.H. *before and after cellulose paint treatment* (Lucilia sericata *eggs*)

Egg no.	Dorsal hatching strip and pleats painted						Ventral side painted			
	1	2	3	4	5	6	7	8	9	10
Before treatment	4·8	5·4	6·1	5·0	7·2	5·0	4·6	6·4	5·5	4·7
After treatment	4·0	2·3	4·6	4·2	5·2	4·6	3·4	3·5	3·9	4·3
Reduction after treatment	0·8	3·1	1·5	0·8	2·0	0·4	1·2	2·9	1·6	0·4

Observation 5. It was earlier mentioned that the decrease in cross-sectional area of eggs with increase in humidity appeared to occur mainly by inward movement of the dorsal side of each egg—the side bearing the hatching pleats. This may have been due to the fact that the contraction across the hatching strip was relatively greater than that of the unspecialized chorion surrounding the rest of the short-axis circumference of the egg. Measurements of the width of the hatching strip were obtained, but the accuracy of the results was so low (the distance involved is but 50 μ or so) that the above possibility could not be verified. The idea is given support, however, by the following observation. Imperfect eggs of *Lucilia caesar* were observed, in which the hatching strip and pleats were very short and extended for only one-tenth of the egg length from the anterior end, leaving the rest of the egg covered entirely by unspecialized chorion of uniform thickness. It was found that in dorsal and lateral views the reduction in diameter with rise in R.H. appeared to occur in these eggs by an inward movement of the whole of the circumference to about the same extent all round the egg.

Observation 6. At 0% R.H. it was observed that the hatching pleats stood nearly upright from the egg surface, and that with increasing humidity they leaned progressively inwards towards each other, so that in saturated air they lay at an angle of 30–40° with the egg surface. These changes are shown in Fig. 3 as diagrammatic sections across the hatching pleats. This movement of the pleats increased the difficulty of measuring the width of the hatching strip with any accuracy, but the movements would appear to involve a decrease (at high humidity) in the distance between the outer edge of the two pleats (distance *AB* in Fig. 3). If this is so, movement of pleats provides a possible mechanism whereby the short axis circum-

ference of the egg may become reduced with increasing humidity, with only a fairly small contraction of the unspecialized chorion, since pleat movements would 'take up the slack'.

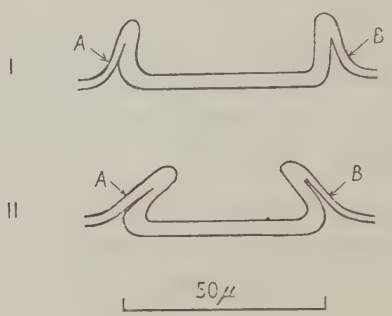


Fig. 3. Diagram of hatching-pleat movements; I, at 0% R.H.; II, at 100% R.H.

The movements of the pleats with humidity may also explain why the chorion usually ruptures on hatching, along the outer margin of either of the pleats, without the necessity of postulating the existence of lines of weakness there. With such movements, the strain would be expected to be greatest at these positions.

The anterior localization of the chorion split during hatching, in contrast to its haphazard occurrence along the length of the pleats when eggs were subjected to sudden large humidity changes (p. 439), is certainly due to the fact that the effects of larval movement are confined to the front end of the shell. When eggs in humid air were observed under the microscope, immediately prior to hatching, the larva could be seen to hammer on the inside of the micropylar plate. This caused the whole of the anterior end of the egg to elongate slightly, thus reinforcing, at the front end only, the strains set up in the shell by elongation of the egg due to high humidity.

Observation 7. When a small perforation was made through the chorion and the vitelline membrane of an otherwise intact egg, a small drop of yolk appeared over the hole. This showed that at room humidity the yolk contents were held under slight pressure by the egg membranes. If the egg was then exposed to increased humidity, the drop of yolk grew larger, but when the humidity fell to its original level, it decreased to its former size by flow of liquid yolk back into the egg. A drop of yolk on a slide did not change size when measured quickly at the two humidities employed above. Thus the egg volume must have been slightly reduced as the humidity increased. It follows therefore that in an intact egg an increase in humidity results in an increase in internal pressure.

Observation 8. If the hatching pleats and strip were entirely removed from an egg at room humidity, the egg became slightly shorter than before, while the gap between the free edges of the chorion became slightly wider than the total width of the hatching pleats and strip removed. This indicated that the chorion was under some transverse (i.e. around the short axis of the egg) tension at room humidity. When such eggs were exposed to saturated air, the gape in the chorion became narrower. This latter phenomenon is equivalent to the curling movements of chorion fragments with humidity changes, described by Davies (1948). It has since been

observed that these curling movements of chorion fragments occur predominantly around the short axis circumference of the egg, and only to a negligible degree along the long axis. This fits in with the above observation of the closing of the longitudinal gape in the chorion with increasing humidity, and with the following observation that a split in the chorion running part of the way round the short axis of an egg, did not show pronounced opening and shutting with changing humidity.

Observation 9. The lengths of eggs were determined at 0 and 90% R.H. at both 17 and 37° C. The increase in length at the high humidity over that at the low was found to be the same at both temperatures, variations being within the range of error ($\pm 6\mu$) of the measuring technique. It seems, therefore, that the shape changes are little influenced by temperature, humidity alone being the governing factor.

Observation 10. The Muscid egg chorion was seen to be stiffer at all humidities below saturation than in saturated air itself. Vacated shells in saturated air collapsed and became closely applied to the substratum. At 90% R.H. and lower, vacated shells did not collapse. In saturated air it was observed that the chorion was limp and that after initial splitting the two sides of the split could readily be forced apart by the hatching larva. In the hatching at 30° C., larvae usually escaped from the shell within 2 min. of initial chorion rupture. At 80% R.H. at the same temperature, larvae were seen to have difficulty in forcing the two sides of the split apart, and took 3–20 min. to escape entirely after initial rupture of the shell. The greater stiffness of the chorion at humidities below saturation does not however account for the effects of humidity on hatching. Examination of large numbers of eggs containing fully developed larvae which had failed to hatch at 70–90% R.H. showed that in no case had the chorion been ruptured. The limiting factor appears to have been the difficulty of initially rupturing the chorion, and not the difficulty of escaping from the shell after rupture had been accomplished at these humidities.

DISCUSSION

The above observations show that the chorion alone is responsible for the humidity-dependent shape changes in Muscid eggs, and further analysis of the mechanism whereby they are accomplished depends on a knowledge of its structure. Davies (1948) showed that chorion fragments underwent curling movements dependent on humidity and ascribed them to the two-layered nature of that membrane indicated by the fact that the outer part of its thickness could be tanned by *p*-benzoquinone, while its inner part could not. The two layers are probably differentially affected by water absorption, leading to expansion of the outer layer around the short axis of the egg with rising humidity. That water absorption is involved is supported by the fact that the shape changes were little affected by temperature. The almost instantaneous nature of the curling movements of chorion fragments, and of the egg shape changes, with humidity change, means that the chorion must contain extremely hydrophilic protein, with a large surface area in relation to volume. The latter condition is fulfilled by the outer chorion layer whose thickness is but 2–3 μ (Davies, 1948) and with an external surface area of about 8600 μ^2 in *L. sericata* eggs. Since oily materials do not spread over it (Davies, unpublished observations) it is also hydrophilic.

Since the two layers appear to behave differentially in changing humidities, the layer of dark bodies embedded between them described by Davies (1948), possibly counteracts shear strain at the interface. The outer layer may be constructed of long protein molecules, producing a structure affected by humidity in a way comparable with mammalian hair- and wool-keratin as described by Astbury & Woods (1931, 1933). No data by X-ray methods are available for the Muscid egg chorion. When pieces of chorion are viewed in polarized light, they are seen to be anisotropic, suggesting that there is no marked parallel orientation of long molecules through most of its thickness. Evidence exists (Davies, unpublished observations) that the inner chorion layer is of a spongy nature, the cavities of the sponge forming a continuous gas space comparable to that shown to exist in the *Rhodnius* egg by Tuft (1950). With this spongy structure, the inner layer may readily 'give' under stress from the outer layer when the latter changes its length and curvature with humidity.

These observations on shape changes may be linked with the hatching process of Muscid eggs as follows: with increasing R.H. from 0%, the cross-sectional area is reduced while the egg elongates. Whether cross-sectional area reduction and egg elongation are respectively cause and effect or vice versa is difficult to say. However, the former process will set up strains in the chorion, liable to result in longitudinal rupture and not rupture around the short axis of the egg. That this was so, was shown by the observation that in eggs subjected to sudden transfer from 0 to 100% R.H., the chorion ruptured along the outer margin of either hatching pleat. Since the amount of elongation of eggs is almost certainly a measure of the cross-sectional area reduction, this amount is also a measure of the strain on the chorion. The curves in Fig. 1 (p. 439) are thus indicative of the progressive increase in strain on the chorion with increasing humidity. They show therefore an increasing probability of successful rupture of the chorion by the enclosed larva, with increasing R.H. From Fig. 1 it is to be expected that at 90–100% R.H. the chorion is under maximum strain owing to the shape changes, and blows by the larva on the inside of the anterior end of the egg are likely to cause rupture fairly quickly. With lower R.H. the strain owing to the shape changes will be less, and many more blows by the larva will be required to produce rupture. Exhaustion or death by desiccation frequently supervenes before hatching has been achieved. This is perhaps the explanation of how at certain humidities below saturation, Muscid eggs show a reduced percentage hatch and a greater dispersal of hatching times in groups of eggs, recorded for *Lucilia sericata* eggs by Davies (1948) and for the eggs of certain dung-breeding Muscids by Larsen (1943). At these humidities all the eggs of the various species may contain fully developed larvae, showing that failure or delay in hatching is caused by some mechanical effect on the shell.

SUMMARY

Certain Muscid eggs are shown to change their shape almost simultaneously with changes in the relative humidity of the air in their immediate vicinity. Evidence is produced that these shape changes are caused by the effect of humidity on the chorion. The net effect of the shape changes appears to be the setting up of in-

creasing strain in the chorion, making it progressively easier for the larvae to rupture their shells, with increasing humidity. These shape changes therefore provide an explanation for certain well-known humidity effects on the hatching of Muscid eggs.

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THE INFLUENCE OF WATER AND LIGHT UPON THE PIGMENTARY SYSTEM IN THE COMMON FROG *RANA TEMPORARIA*

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(With Eight Text-figures)

INTRODUCTION

Many factors, both internal and external, influence the chromatic changes of cold-blooded vertebrates, and of these, three external variables are recognized as significant—light, heat and moisture.

The frog can change its colour, from brown or black through intermediate tints to pale lemon, by changing the contraction and expansion of three types of chromatophores—the epidermal and dermal melanophores and the xantholeucophores. The activity of the dermal melanophores, however, dominates these macroscopic changes. It is already established that an illuminated light background, dryness and moderate temperatures, favour the pallor associated with the contraction of the dermal melanophores, whilst an illuminated dark background, moisture and low temperatures induce their expansion giving darkening.

The influence of light has received most attention, 'indeed almost the whole of the available data based on refined measurements of the behaviour of the dermal melanophores refers to the influence of light' (Hogben, 1942). Our knowledge of the effect of moisture is based more upon general observations than critical experiments, and still less is known of the combined influence of two or more factors.

Hogben (1924) concludes that one of the most important factors involved in colour response of adult Anura is moisture—humidity promoting darkening. However, the relative importance of these external agents appears to vary from group to group and between different species of the same groups. Thus Parker & Scatterty (1937) see no reason to assume that with the spotted frog, *Rana pipiens*, either differences in temperature or humidity (jars dry—jars 10 cm. of water) played any part whatsoever in the final responses.

More precise information therefore is needed about the effect of moisture upon the dermal melanophores, both as an independent agent and in relation to other factors.

MATERIALS AND METHOD

Frogs (*R. temporaria*) from various sources were used during these investigations. They were sorted out according to weight and sex and kept under shady moist conditions until required. Only those in good condition were used for the experi-

ments, during which time they were not fed. After use for 6–8 weeks each group of animals was discarded. Under these conditions no deaths occurred. The experiments were carried out upon a bench illuminated by four 60 W. pearl bulbs fitted with white reflectors (27 cm. in diameter), hanging 45 cm. from the bench for studies of the effect of illuminated backgrounds, or in a dark chamber for studies of reactions in the absence of light.

To produce white or black backgrounds the whole of the insides of jars and bins used as containers for the animals during the experiments were painted with white enamel (light scattering) or black paint (matt finish, light absorbing), and when assembled on the bench under the light they were surrounded by white or black screens according to the experimental requirements.

Four conditions were used with respect to moisture—dry, 'moist', 'half-immersed', and immersed. In the first three of these conditions the frogs were placed singly in either white or black walled jars (7 cm. in diameter and 12 cm. in depth), with gauze tops, secured by an elastic band.

(*a*) In dry conditions surface water was removed from the frogs, which after evacuation of the bladder were placed in dry jars.

(*b*) In 'moist' conditions the frogs were placed in approximately 5 c.c. of water in the jars—sufficient to damp the walls with a film of water on the floor, but leaving the dorsal surface of the frog comparatively dry, although the closed space would be humid.

(*c*) In 'half-immersion', the frogs were placed in jars with sufficient water added to cover the hind-limbs and the posterior half of the dorsal surface.

(*d*) In 'immersed' conditions frogs were placed in a large bin ($28 \times 41 \times 33$ cm.) in groups of 8–15, filled with water to a depth of 18–20 cm. To avoid complications due to a lowering of the oxygen supply, the water was changed hourly when necessary during which time the oxygen content of the water was not found to change significantly.

The temperature of the water was maintained the same as that of the dry jars, the variation of temperature never exceeded 2° C. in a single day.

The degree of expansion of the dermal melanophores in the web between the 3rd and 4th digit of the left hind-limbs was observed under the microscope, and the degree of expansion assessed numerically using the scale introduced by Hogben & Gordon in 1930: (1) punctate; (2) stellate-punctate; (3) stellate; (4) reticulate-stellate; (5) reticulate. There is considerable uniformity in the behaviour of the individual melanophores in the web, and they rarely differ over more than one unit of the scale. When differences existed the scale units were modified as follows: all punctate, 1; punctate with a few stellate-punctate, 1·25; half-punctate and half-stellate-punctate, 1·5; stellate punctate with a few punctate, 1·75.

The melanophore condition of the frogs in any group were observed at known intervals and the average index ($\bar{\mu}$) plotted against time. The graphs obtained under the various experimental conditions facilitated and standardized comparisons. To eliminate discrepancies due to individual variations, a further refinement was employed. A number of frogs of the same sex and approximate weight were divided

into four groups for testing the reaction to moisture in relation to any particular condition of illumination. On four successive days the groups were reversed so that each group in turn had been subjected to the same condition. The temperature varied little in any 4 days (2–3° C.). At the end of this period, graphs of the total number under all the experimental conditions could be plotted. In this way since each graph represents the same frogs any trends noted would be significant and not due to the variability of individual response.

The two extremes, black illuminated half-immersed and white illuminated dry, were used throughout for obtaining an initial equilibrium of the melanophores. In practice these two sets of conditions (degree of illumination and temperature being the same in each case) were satisfactory though not theoretically optimum—the overwhelming majority of specimens responding with $\mu = 1$–$1{\cdot}75$ to white illuminated dry conditions and $\mu = 5$ to black illuminated half-immersed conditions.

OBSERVATIONS

(*a*) *General*

In general it was found that when dark frogs ($\mu = 5$) were removed from water but retained on a black illuminated background, they showed no immediate chromatic response, but that on the addition of water to pale individuals ($\mu = 1$) on a white illuminated background an immediate response was obtained, the melanophores expanding to varying degrees. There were, however, indications that with time the melanophores of frogs in dry surroundings would contract even in a black illuminated environment.

It was further found that complete immersion upon a white illuminated background resulted in some specimens reaching $\mu = 5$ in the first hour or so, a condition which could be maintained for a considerable period (in the tables the number of frogs having $\mu = 5$ at each interval is recorded). But whereas a black illuminated wet environment almost without exception produces a reticulate condition, a white illuminated immersed environment produces partial darkening in almost all specimens but only a proportion attain the maximum expanded state.

(*b*) *Illumination in relation to water*

Illumination apparently plays a significant part in the darkening due to immersion. Thus upon immersion the time taken for frogs to come to equilibrium with the environment is greater in darkness than in a white illuminated environment which would in the absence of water provoke contraction, i.e.

$$\mathrm{WID}\,^{\mathrm{T}}\mathrm{WII} < \mathrm{WID}\,^{\mathrm{T}}\mathrm{DI}$$ (Fig. 1, Table 1)

(WID = white illuminated dry; WII = white illuminated immersed; DI = dark immersed).

The determination of significant differences in relation to increasing amounts of water (i.e. increasing degrees of immersion) was studied throughout the seasons upon successive groups of frogs. It was observed that the variation of response to water was much greater than that due to background.

The results with winter specimens upon a white illuminated background (Fig. 2) show some correlation between the melanophore expansion achieved and the amount of water present—the greater the amount of water the greater the average degree of expansion. (These averages derive their differences from two sources –the number

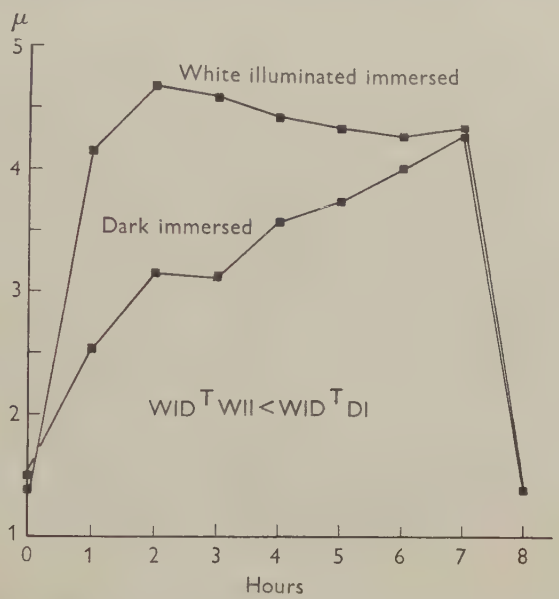


Fig. 1. Autumn frogs (♂♂, $n=36$). Temp. 15° C. White illuminated: dry (16 hr.) → immersion → white illuminated: dry (1 hr.).

Table 1. *Autumn frogs. Temp.* 15° C. *Males* ($n=36$)

White illuminated: dry (16 hr.)→immersed (7 hr.)→white illuminated: dry (1 hr.)

Background	Immersed								WID
	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	7 hr.	8 hr.
White illuminated: $\bar{\mu}$*	1·39	4·16	4·69	4·61	4·44	4·35	4·29	4·37	1·39
$\mu=5$	0	20	24	24	19	19	18	18	0
σ	0·44	0·92	—	—	—	—	—	0·75	—
Darkness: $\bar{\mu}$	1·5	2·53	3·17	3·13	3·59	3·76	4·04	4·32	1·39
$\mu=5$	0	3	10	10	10	14	15	23	0
σ	0·41	1·30	—	—	—	—	—	0·85	—

$\bar{\mu}$*=average melanophore index. σ=standard deviation. ($\mu=5$)=no. of specimens in the group reaching maximum expansion.

of frogs in each group which gave a response to water and the degree of their melanophore expansion.) The effect of the degree of immersion upon the paling of initially dark specimens (Fig. 3) confirms these findings. The gradients of the curves show a parallel correspondence, but too much significance should not be attached to this particular evidence of the effect of water owing to the arbitrary nature of the numerical system employed.

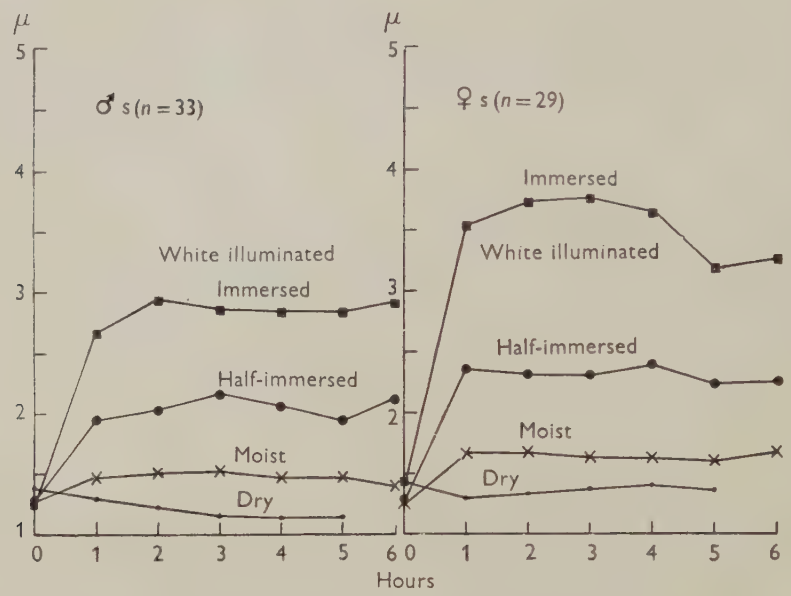


Fig. 2. Winter specimens. Temp. 16–18° C. White illuminated: dry (16 hr.) → white illuminated: degrees of immersion.

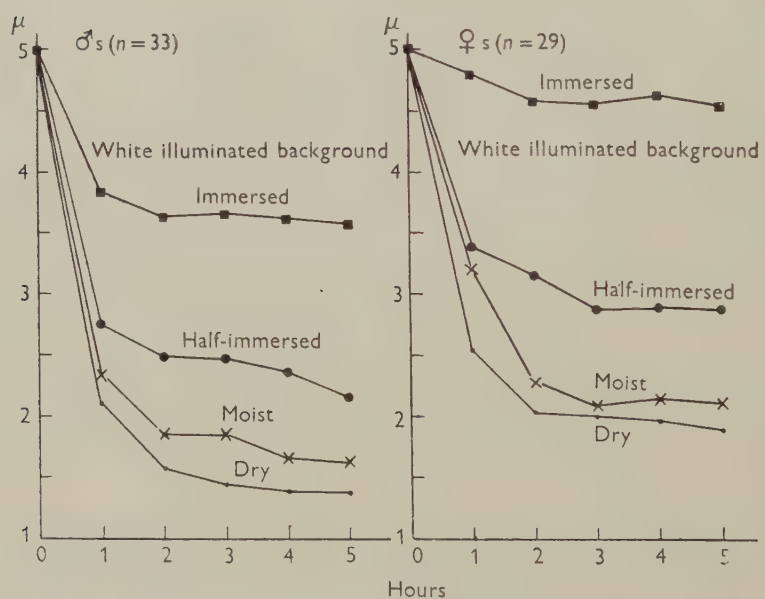


Fig. 3. Winter specimens (same frogs as in Fig. 2). Temp. 15–18° C. Black illuminated: half immersion (16 hr.) → white illuminated: degrees of immersion.

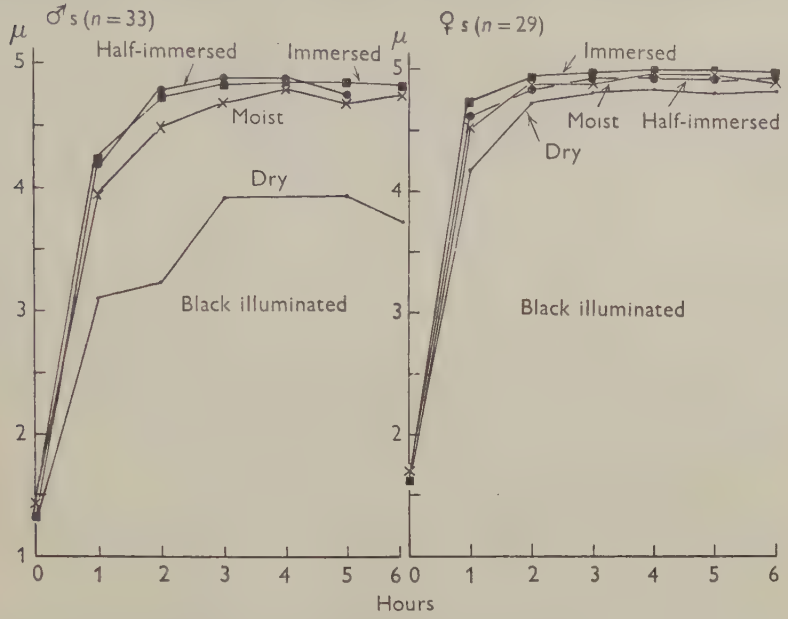


Fig. 4. Winter specimens (same specimens as Figs. 2, 3). Temp. 16° C. White illuminated: dry (16 hr.) → black illuminated: degrees of immersion.

Table 2. *Winter specimens (same frogs as in Figs. 2 and 3)*

White illuminated: dry (16 hr.)→Black illuminated: degrees of immersion.

Black illuminated	Males ($n=33$)						
	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.
Immersed: $\bar{\mu}$	1·33	4·24	4·75	4·85	4·85	4·87	4·84
$\mu=5$	0	4	22	24	27	27	28
Half-immersed: $\bar{\mu}$	1·32	4·21	4·8	4·9	4·9	4·77	—
$\mu=5$	0	4	24	27	29	28	—
Moist: $\bar{\mu}$	1·43	3·94	4·5	4·7	4·83	4·7	4·78
$\mu=5$	0	3	19	20	21	21	24
Dry: $\bar{\mu}$	1·36	3·1	3·24	3·93	3·95	3·96	3·74
$\mu=5$	0	2	2	8	10	5	4
σ	—	—	—	—	—	—	0·88

Black illuminated	Females ($n=29$)						
	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.
Immersed: $\bar{\mu}$	1·63	4·72	4·94	4·98	5·00	5·0	5·0
$\mu=5$	0	15	27	28	29	29	29
Half-immersed: $\bar{\mu}$	1·60	4·61	4·84	4·94	4·94	4·94	4·94
$\mu=5$	0	14	22	27	27	27	27
Moist: $\bar{\mu}$	1·69	4·53	4·86	4·89	4·96	4·98	4·93
$\mu=5$	0	15	25	26	28	28	27
Dry: $\bar{\mu}$	1·69	4·18	4·72	4·82	4·85	4·83	4·85
$\mu=5$	0	3	19	21	20	22	23
σ	—	—	—	—	—	—	—

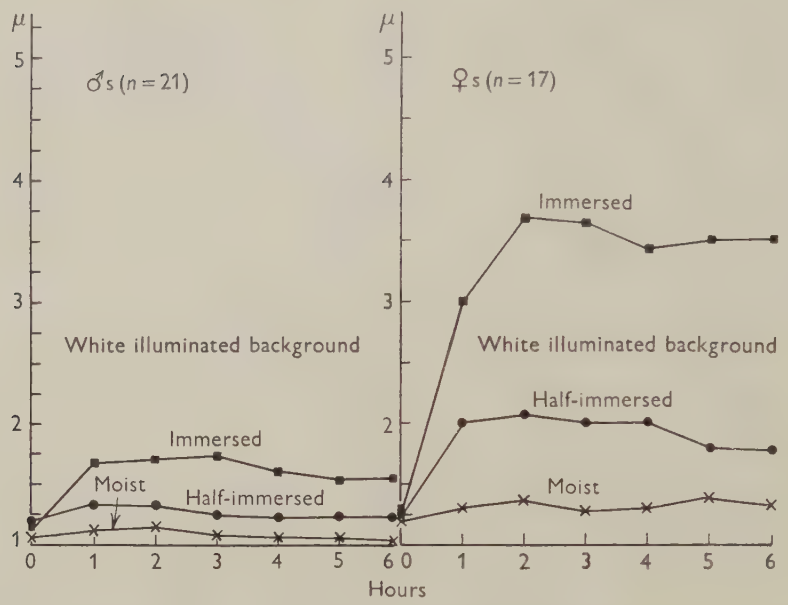


Fig. 5. Breeding specimens. Temp. 14° C. White illuminated: dry (16 hr.) → white illuminated: degrees of immersion.

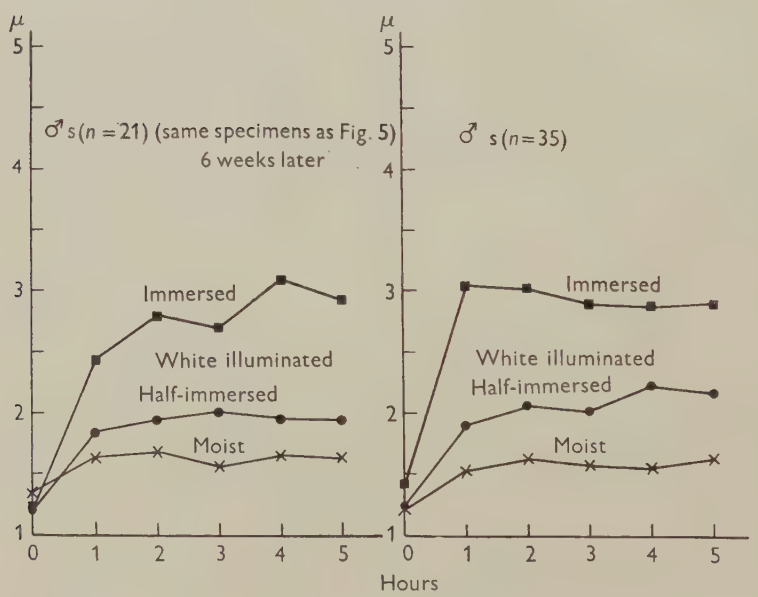


Fig. 6. Post breeding specimens. Temp. 15° C. White illuminated: dry (16 hr.) → white illuminated: degrees of immersion.

It would seem that the breeding cycle interrupts the normal response to water only in the *males* (Fig. 5.) With the onset of the breeding season the response declines, whilst at its height the darkening response to any degree of immersion fails to be elicited. As the breeding season passes (naturally in the field or artificially in the laboratory) water again asserts itself as a co-dominant factor in the adjustment of the melanophores to the environment (Fig. 6).

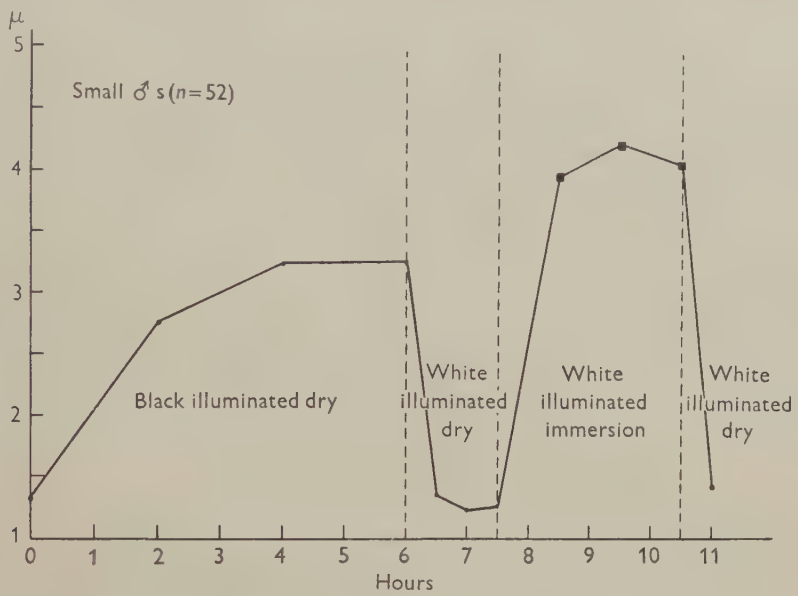


Fig. 7. Summer specimens. Temp. 18–19° C. White illuminated: dry (16 hr.).

Table 3. *Summer specimens. Temp.* 18–19° C. *Small males* ($n=52$)

White illuminated: dry (16 hr.)→black illuminated: dry→white illuminated: immersed.

	BID (6 hr.)				WID (1½ hr.)			WII (3 hr.)			WID (½ hr.)
	0 hr.	2 hr.	4 hr.	6 hr.	6½ hr.	7 hr.	7½ hr.	8½ hr.	9½ hr.	10½ hr.	11 hr.
$\bar{\mu}$	1·31	2·76	3·26	3·27	1·35	1·23	1·26	3·99	4·24	4·09	1·43
σ	0·35	1·21	—	1·42	0·40	—	0·35	1·18	—	1·28	0·65
$\mu=5$	—	7	—	16	—	—	—	21	—	24	—

Water exerts its maximum effect upon the melanophores in summer when the greatest degree of expansion can be obtained upon a white background by immersion, and the greatest degree of contraction upon a black background by desiccation (Fig. 7, Table 3). This can be seen especially when Fig. 2 is compared with Fig. 7, and the difference in the degree of expansion to immersion upon a white background is appreciated.

A black illuminated background obscured any expansion of the melanophores due to the amount of water present (Fig. 4, Table 2)—the reticulate condition was obtained in all cases more or less simultaneously. In dry conditions, however, in

general $\mu < 5$ except in the case of gravid females (Table 4). Desiccation on a black background induces contraction, but at the end of any prolonged period of dryness there was a large individual variation ($\mu = 1{\cdot}5$–$5{\cdot}0$). This contraction was shown more readily in small specimens as would be expected, and in summer more readily than winter, whilst with females distended with eggs even after 80 hr. there was no appreciable contraction.

Table 4. *Winter specimens. Temp.* 18·5° C.

White illuminated: dry→black illuminated: dry.

Time previously dry	0 hr.	2 hr.	4 hr.	8 hr.	20 hr.	22 hr.	24 hr.
4 hr. (males, $n=10$): $\bar{\mu}$	1·0	4·15	4·80	4·50	4·30	4·05	3·75
$\mu=5$	0	6	9	6	6	6	5
							1·39
8 hr. (males, $n=10$): $\bar{\mu}$	1·0	3·45	3·9	4·30	3·45	3·20	3·15
$\mu=5$	0	3	4	4	4	4	3
							1·51
12 hr. (males, $n=10$): $\bar{\mu}$	1·0	4·7	4·95	4·15	—	—	3·15
$\mu=5$	0	7	9	3	—	—	1
							1·23
24 hr. (males, $n=10$): $\bar{\mu}$	1·0	3·22	3·75	3·02	—	—	2·32
$\mu=5$	0	2	3	3	—	—	0
							1·34

Gravid females ($n=10$).

White illuminated: dry (24 hr.)→black illuminated: dry.

	0 hr.	2 hr.	4 hr.	8 hr.	20 hr.	22 hr.	24 hr.	46 hr.	50 hr.	54 hr.
$\bar{\mu}$	1·2	4·3	4·72	4·72	—	—	4·97	5·0	4·95	4·45
$\mu=5$	0	1	6	6	—	—	9	10	9	8

While large individual differences existed it would appear that:

(1) The expansion response to a black illuminated dry environment is unstable, prolonged drying inducing a reduction in the melanophore expansion initially achieved.

(2) The length of the initial dry period before transference to a black background effects this expansion—the degree attained roughly varying inversely with the length of the initial white dry period (Table 4).

(*c*) *Darkness in relation to water*

The response in winter and summer to any degree of water in darkness was a gradual but complete expansion of the dermal melanophores (Table 5). Where initially expanded, this expansion was in general maintained (Table 6). The responses were somewhat erratic, and some diurnal as well as individual variation was evident.

During the breeding season some peculiarities were shown by both *males* and *females* especially to complete immersion in darkness. Initially dark frogs paled rapidly when immersed in darkness (whilst pale specimens remained so for a number

Table 5. *Summer specimens. Temp.* 17·5° C. *Males* (13 *specimens in each group*)

White illuminated: dry (16 hr.)→darkness: degrees of immersion.

Degree of immersion	0 hr.	2 hr.	6 hr.	8 hr.	24 hr.	26 hr.	28 hr.	30 hr.	32 hr.	48 hr.
Immersed: $\bar{\mu}$	1·42	3·42	4·70	4·94	4·80	4·80	4·84	5·00	4·70	4·80
$\mu=5$	0	3	10	12	11	11	10	13	9	11
Half-immersed: $\bar{\mu}$	1·52	4·4	4·94	4·88	4·90	4·94	4·92	4·96	4·84	4·92
$\mu=5$	0	7	11	12	9	11	11	12	10	11
Moist: $\bar{\mu}$	1·4	3·3	4·27	4·54	4·21	4·3	4·5	4·8	4·36	4·4
$\mu=5$	0	4	7	8	6	7	9	10	8	9
Dry: $\bar{\mu}$	1·46	2·27	3·25	3·27	3·73	4·05	3·53	3·79	3·53	3·0
$\mu=5$	0	0	0	2	3	5	4	4	4	1
σ	0·32	0·87	—	—	—	—	—	—	—	1·13

Table 6. *Summer specimens. Temp.* 18–20° C. *Males*

Black illuminated: half-immersed→darkness: degrees of immersion.

Darkness	0 hr.	2 hr.	4 hr.	6 hr.	8 hr.	10 hr.	24 hr.
Immersed ($n=8$): $\bar{\mu}$	5·0	5·0	5·0	5·0	5·0	5·0	5·0
$\mu=5$	8	8	8	8	8	8	8
Half-immersed ($n=8$): $\bar{\mu}$	5·0	4·28	4·9	4·84	4·93	4·93	5·0
$\mu=5$	8	5	7	7	7	7	8
Moist ($n=7$): $\bar{\mu}$	5·0	4·75	4·85	4·46	4·71	4·21	4·71
$\mu=5$	7	5	6	5	6	5	5
Dry ($n=7$): $\bar{\mu}$	5·0	3·07	3·1	3·25	3·64	2·75	2·78
$\mu=5$	7	1	1	2	3	2	1
σ	0·0	1·29	—	—	—	—	1·42

Darkness	26 hr.	30 hr.	34 hr.	36 hr.	48 hr.	56 hr.	72 hr.
Immersed (n=8): $\bar{\mu}$	5·0	5·0	5·0	5·0	5·0	5·0	5·0
$\mu=5$	8	8	8	8	8	8	8
Half-immersed ($n=8$): $\bar{\mu}$	5·0	5·0	5·0	5·0	5·0	5·0	5·0
$\mu=5$	8	8	8	8	8	8	8
Moist ($n=7$): $\bar{\mu}$	4·85	4·89	4·85	4·85	5·0	5·0	5·0
$\mu=5$	6	6	5	5	7	7	7
Dry ($n=7$): $\bar{\mu}$	3·03	3·68	3·35	3·57	4·14	3·57	4·28
$\mu=5$	1	2	3	2	1	2	3
σ	—	—	—	—	—	—	—

Table 7. *Breeding females. Temp.* 17·5° C.

Black illuminated: half-immersed→darkness: degrees of immersion.

Darkness	0 hr.	4 hr.	8 hr.	20 hr.	24 hr.	28 hr.	32 hr.	44 hr.	48 hr.
Immersed ($n=6$): $\bar{\mu}$	5·0	2·79	1·33	2·83	4·25	3·2	2·29	3·08	4·41
$\mu=5$	6	1	0	0	2	0	0	1	1
Half-immersed ($n=6$): $\bar{\mu}$	5·0	5·0	4·04	5·0	5·0	4·97	4·5	5·0	5·0
$\mu=5$	6	6	2	6	6	5	4	6	6
Moist ($n=6$): $\bar{\mu}$	5·0	3·79	2·79	5·0	4·87	4·75	4·58	5·0	5·0
$\mu=5$	6	3	1	6	4	3	2	6	6
Dry ($n=6$): $\bar{\mu}$	5·0	3·5	1·7	4·95	3·66	4·66	3·62	4·82	4·62
$\mu=5$	6	1	0	5	2	2	0	4.	3

of hours), but eventually the melanophores expanded (Table 7). This paling did not appear to be due to the shock of immersion since it was apparent after 5 hr. adjustment to immersion on a white illuminated background and, moreover, after a prolonged period in a dark dry environment, immersion resulted in the immediate expansion of the melanophores in the normal way. Apart from these initial peculiarities the breeding season gave results comparable to other seasons in both sexes, in that water to any amount eventually induced extreme melanophore expansion. Individual specimens gave more widely varying responses than at other seasons.

Table 8. *Winter males. Temp.* 16–17° C. ($n=41$)

Damp shady conditions→Dark: dry.

μ	1 and 1·25	1·5	2·0	2·5	3·0	3·5	4·0	4·5	5·0
67 hr.	—	6	2	4	5	2	5	2	15
72 hr.	1	6	5	3	6	1	4	5	10
92 hr.	3	8	2	3	5	1	8	3	8
120 hr.	6	6	7	3	2	1	4	3	9

There was a considerable range of response to dark dry conditions, and within a sufficiently large group even after several days a full range of melanophore indices from $\mu=1$–5 was obtained. However, there was a tendency towards contraction as the period of dryness lengthened (Table 8).

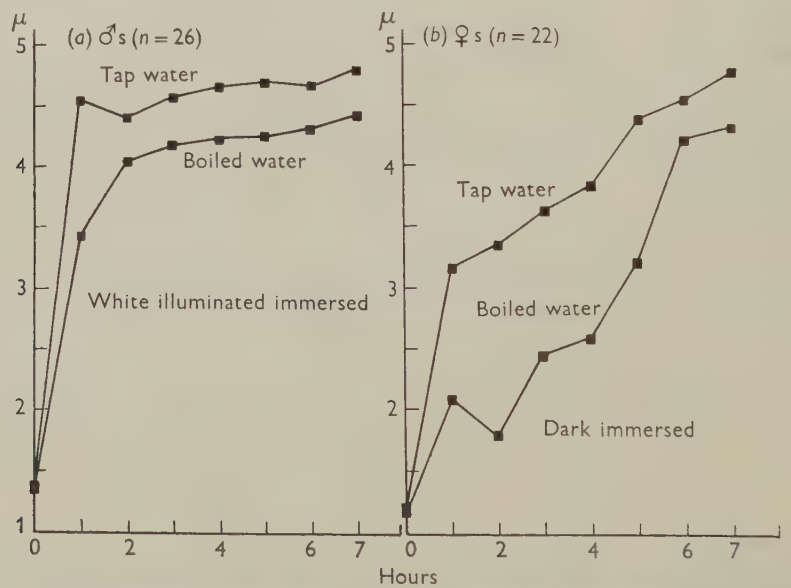


Fig. 8. Summer males. Temp. 23·5° C. White illuminated: dry (16 hr.) → immersion.

(*d*) *Oxygen content of water in relation to melanophore responses*

Hogben (1924) declares that oxygen deficiency induces the expansion of the dermal melanophores, and since the oxygen tension of water is lower than the air the question arose whether darkening in water was the result of oxygen deficiency.

Table 9. *Summer males. Temp.* 23·5° C.

White illuminated: dry (16 hr.)→immersion.

Immersion	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	7 hr.
	(*a*) White illuminated background ($n=26$)							
Tap water: $\bar{\mu}$	1·38	4·56	4·39	4·58	4·66	4·7	4·65	4·8
$\mu=5$	0	19	18	20	20	19	18	22
σ	0·41	0·92	—	—	—	—	—	0·52
Boiled water: $\bar{\mu}$	1·35	3·43	4·04	4·18	4·23	4·24	4·29	4·42
$\mu=5$	0	7	10	12	13	11	11	17
σ	0·43	1·6	—	—	—	—	—	0·88
	(*b*) Darkness ($n=22$)							
Tap water: $\bar{\mu}$	1·22	3·18	3·36	3·64	3·84	4·39	4·56	4·78
$\mu=5$	0	6	6	4	10	14	16	17
σ	0·17	1·59	—	—	—	—	—	0·57
Boiled water: $\bar{\mu}$	1·18	2·1	1·8	2·48	2·51	3·23	4·24	4·32
$\mu=5$	0	1	0	1	2	5	10	12
σ	0·17	0·98	—	—	—	—	—	0·93

Animals were immersed in water which had been boiled to reduce the oxygen content ($\doteq 50\%$). No significant differences were observed in the responses to immersion in this boiled and fresh water upon a black illuminated background, but upon a white illuminated background and in darkness the responses were slower in boiled water although the equilibrium points were the same (Fig. 8, Table 9). The oxygen content was checked at hourly intervals using Winkler's method. Increasing the oxygen content by bubbling oxygen through the tank (250% increase maintained) had no significant effect upon the melanophore responses.

Thus if the lack of oxygen was the cause of darkening due to immersion, a more rapid darkening should follow the lowering of the oxygen still further by boiling, and a less rapid darkening (if any) when it was increased. This was not so, however, so that the cause of darkening due to water requires another explanation.

DISCUSSION

All animals capable of chromatic response respond to photic stimulation. Indeed light, if not the sole agency, is certainly the dominant one in aquatic animals, but temperature and moisture also play a considerable part in the behaviour of terrestrial species. Immersion produces darkening, masking a white background effect and making moisture dominant.

Of the photic responses there is a primary response independent of the eyes, and a secondary response which is superimposed upon the primary response in the adult, which is ocular. Contrary to the secondary response, the primary induces contraction in darkness and expansion in the light. It is suggested that this response is due to the direct stimulation of the melanophores, and it reappears in the adult when the vision is destroyed.

There are at least two independent physical factors affecting the expansion of the melanophores in the normal adult—a black illuminated environment and moisture.

Individually each is capable of causing maximum expansion. In the production of extreme expansion a black illuminated moist environment is more generally effective and immediate than a dark moist, which is more effective than black illuminated dry, which at best induces an unstable expansion, continued drying causing contraction.

With regard to moisture, Hogben (1942), summarizing the evidence, arrives at two conclusions concerning the mode of action of moisture on the chromatic responses: (*a*) low humidity beyond a certain limit either abolishes the primary response to light or inhibits the stimulation of the B-area of the eye; (*b*) the action of humidity almost certainly involves the skin receptors.

In these experiments immersion rather than humidity indicating moist conditions is more effective in producing expansion of the melanophores (Fig. 2). It is seen, moreover, that there is a general correspondence between the degree of immersion and the degree of darkening produced. This would support Hogben's statement involving the skin receptors—the greater the area of the skin covered the greater the stimulus for expansion. It must be noted, however, that the original statement referred to humidity, whereas the emphasis here is upon the extent of contact with water.

In darkness frogs as they dry tend to pale—certainly they are paler than moist specimens (Tables 6 and 8). This is inconsistent with the view that low humidity abolishes the primary response. The primary response involves contraction in darkness. Thus it would be expected that moist frogs would be paler than dry frogs in darkness, whilst the reverse is observed.

Again if the primary response is still functioning in the normal adult, the results shown in Fig. 1, Table 1, become intelligible, where the time taken for initially pale frogs to come to equilibrium with immersion upon a white illuminated background was less than that in darkness. With illumination the primary response is working with moisture in inducing expansion, in darkness it is working against. The secondary response is eliminated in darkness and opposes the response on a white background.

It is clear, therefore, that the response due to water must be considered as distinct from the primary response, since the two responses may antagonize or reinforce each other. Water may inhibit to a certain extent the elimination of the active expanding factor (B-factor), since dark frogs immersed upon a white background maintain a greater degree of expansion than that attained by initially pale frogs (Figs. 2, 3). However, as initially pale frogs do darken, water cannot exert its sole effect by its intervention in the elimination of active B-factor.

It is clear that predominantly terrestrial Amphibia are different from the essentially aquatic forms in their chromatic receptor apparatus. The skin has assumed an important role and the eye has receded in importance. With the increased influence of the skin as a receptor organ, temperature and water assume a greater significance.

During the breeding season, the chromatic response to immersion was absent in the males. Frogs return to water during the breeding season, and it appears probable that some change in the internal secretions affects the physiology of the skin and causes a terrestrial animal to become an aquatic one when the photic response

assumes its original supremacy for a time. Why the females should not show the same tendency is obscure.

It may be that in *R. pipiens* (in which Parker & Scatterty (1937) state water induces no chromatic response and which is a marsh-dweller and lives in mud or water throughout the year) the mechanism of moisture response has not been elaborated, or has ceased to be effective unlike the more nearly terrestrial species (e.g. *R. temporaria*).

Immersion of frogs causes the expansion of the dermal melanophores and the uptake of water, both being ascribed to posterior pituitary activity and both apparently involving activity of the skin (Steggarda, 1931). But since there is no available evidence directly correlating the phenomena, since the water-balance principle is elaborated in the pars nervosa and the melanophore stimulant in the pars intermedia (Heller, 1941; O'Connor, 1947). Also whilst it is seen (Fig. 7, Table 3) that the expansion due to immersion takes only 1 or 2 hr. upon an illuminated background, water uptake is considerably longer in achieving its maximal effect.

It is now generally agreed that chromatic responses are not primarily a part of an effective system of protective coloration, and the biological significance of these changes is by no means obvious.

The chromatic responses of the frog may be related to temperature regulation and the conservation of water, and it is possible that the paling due to dryness (and high temperatures) is an attempt to overcome adverse factors in the environment. Thus a dark skin absorbs radiant energy which must then be lost by evaporation of water, whereas a pale skin will reflect radiant energy with the attendant conservation of moisture so essential to the respiratory function of the skin of the frog.

When there is any degree of immersion in water the need for its conservation is removed, and it may be that the resultant darkening upon a white background implies the onset of the inactive state with only the ocular stimulation exerting a contracting effect not sufficient alone to maintain the contracted condition. The dark phase is by many workers assumed to be the inactive state. Parker (1948), however, considers the active state to be exhibited by the motion of the melanin within the melanophores. In this case the presence of water must produce a definite stimulus inducing expansion, and extreme dryness an opposing stimulus inducing contraction.

The effect of water may be due to a reflex action involving the skin receptors or an endocrine co-ordination involving skin receptors and internal secretions as effectors.

Background responses are known to involve an endocrine system with the eye as a receptor, and either one or two internal secretions. The pars intermedia certainly liberates the B-factor, but whether paling is due to a separate W-factor or to inhibition impulses from the eye to the pars intermedia is still a controversial issue—although damage of the nerves of the supra optico-hypophysial tract certainly brings about the permanent expansion of the melanophores.

The same endocrine relations may be operative in the pigmentary responses to water. When the skin reaches a certain desiccation its receptors may send inhibitory impulses to the pars intermedia causing cessation of the liberation of the B-factor

(or alternatively another endocrine agent may be liberated to induce contraction). The slowness of contraction might be due to the reduction in excretion with its attendant reduction in elimination of the B-factor already present.

Immersion upon a white background produces the inactive expanded state of the melanophores, or else there is a positive stimulation of the skin with the result that B-factor is liberated in amounts sufficient to overcome the paling effect of the background response.

Hence although the significance of water in relation to the chromatic changes in the frog have been demonstrated, the mechanism involved is by no means so obvious and only tentative explanations can be offered to account for them. Still less can any definite statements be made regarding the function of these colour changes.

SUMMARY

1. A significant influence of water in relation to colour change in *Rana temporaria* has been established.

2. Its action on the melanophores in relation to background shows it exerts an independent influence. Thus (*a*) with a black illuminated environment, water accentuates the background response, (*b*) with a white illuminated environment there is antagonism, (*c*) in darkness the presence of water induces extreme expansion, and drying induces contraction, but only after prolonged periods.

3. Immersion on a white illuminated background causes immediate expansion, and prolonged drying even upon a black background induces contraction.

4. During the breeding cycle, the males fail to darken when immersed upon a white illuminated background. This particular seasonal effect is not shown by the females.

5. It was further shown that the water response is independent of the primary response.

6. The greater the degree of immersion, the greater the degree of expansion maintained upon a white illuminated background, so that the area of contact is significant, and it is highly probable that skin receptors are concerned.

7. The lower oxygen tension of water as compared with that of the atmosphere was not a significant factor in the expansion of the melanophores due to immersion.

I should like to thank Prof. E. A. Spaul for his advice throughout the investigations and for his constructive criticism of the paper.

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THE SENSORY PHYSIOLOGY OF THE HARVEST MITE *TROMBICULA AUTUMNALIS* SHAW

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(With Twenty-four Text-figures)

INTRODUCTION

The ectoparasitic habit of the hexapod larva of *Trombicula autumnalis* is the cause of much discomfort to residents of infected localities in the British Isles, between late June and the beginning of October. The mite is a member of the Trombiculid group which includes species known to transmit disease in some parts of the world.

The unfed larvae are found either upon the soil or climbing upon low-lying vegetation. Under suitable conditions they aggregate into clusters and are then more easily detected as orange patches. Development to the nymphal stage cannot take place unless the larvae obtain a meal from the superficial tissue of a vertebrate host to which they must securely attach themselves. The nymphs and adults are non-parasitic and lead a hypogeal existence at a depth of about 12 in. below the surface of the soil (Cockings, 1948).

The hairs of a mammal, or the feathers of a bird, as they brush against infected soil or low-lying vegetation, are admirably suited for picking up the mites, but the question arises, to what extent are sensory perceptions of environmental stimuli of the mites directed towards the acquisition of a host. The chief aim of the present work has therefore been to investigate (*a*) the responses of the mite to stimuli most likely to have value with respect to the problem of acquiring a host, and (*b*) the nature of the sensory organs.

Few workers have studied the orientation mechanisms of members of the Acarina. Henschel (1929) described the reactions of *Tyrolichus casei*, the cheese mite, to chemical stimulation. Totze (1933) studied the sheep tick, *Ixodes ricinus*, and Solomon (1937) the red-legged earth mite, *Halotydeus destructor*, in relation to environmental conditions. Lees (1948) reinvestigated the reactions of *Ixodes ricinus* with respect to those stimuli the tick will encounter in its natural environment. As to the behaviour of harvest mites there exist only a few scattered and very incomplete references to observations in the field. It was found desirable to carry out a preliminary ecological study of *Trombicula autumnalis* in 1947 to provide information upon which to base the present work. The observations of the mite in the field were invaluable for suggesting lines of investigation in the laboratory, and for assessing the significance of the sensory perceptions of the mite with respect to acquiring a host.

REACTIONS TO LIGHT

Among members of the Acarina, unfed ticks and harvest mites closely resemble each other in showing a tendency to climb upwards, a movement which in some unfed insect larvae is associated with a positive response to light. However, the results of Totze (1933) and MacLeod (1935) on the response to light of eyeless species of ticks are contradictory. Lees (1948) found himself in agreement with MacLeod, who was quite unable to confirm the findings of Totze, who maintained that unfed ticks, *Ixodes ricinus*, in all stages are strongly photopositive. Lees, like MacLeod, tested unfed and engorged ticks which were all photonegative.

Ticks show a strong inclination to climb up to the tips of the stems of rushes and grasses. This behaviour could be accounted for either by a response to light, or to the influence of some form of negative geotaxis. Krijgsman (1937), working with tick larvae, *Boophilus annulatus*, found they were indifferent to gravity. Lees (1948) stated that it was not easy to interpret the results of his gravity tests on *Ixodes ricinus*, but concluded that although negative geotaxis may be of some significance, the inclination to maintain a position at the tips of glass rods, serving as models of natural grass or rush stems, was partly a tactile response following arrival at the tip. If, as the evidence suggests, ticks are photonegative and independent of gravity, it is difficult to account for the persistent nature of their tendency to climb, upwards, which must invariably lead unfed ticks, in the first place, from the roots of vegetation and up the stems of grasses, before they experience arrival at the tip, which as Lees suggests only enhances this behaviour.

The findings of gravity tests on harvest mites (see p. 482) showed that they assumed a random distribution upon a vertical rod. This apparent independence of gravity is also readily shown by the mites when they move about upside down upon the under surface of the window of a light trap (Jones, 1950*a*). With the elimination of gravity as an influence, there remained the possibility of a response to light being responsible for the upward-climbing movement.

Responses to beams of light

Method. The tests were made in a dark room. A circle of filter-paper, 18 cm. in diameter, placed upon a circular glass plate, 20 cm. in diameter, served as the experimental field. Although a dark surface is usually prescribed, preliminary tests showed the reactions of mites to horizontal light to be similar upon either black or white filter-paper. White filter-papers were therefore used because it was easier to mark the paths traversed by the mites. Illumination was provided by 40 W. and 100 W. bulbs enclosed in light-proof containers with a circular aperture $2\frac{1}{2}$ cm. in diameter, to allow the escape of a horizontal beam of light across the filter-paper. The light, before it escaped, was first cooled by passing it through a $2\frac{1}{2}$ in. tank containing an acidified solution of alum. Light intensities were measured with a G.E.C. photometer.

Single horizontal beam. Mites were placed, usually three at a time, in the centre of the illuminated field. On exposure to a strong beam, with a light gradient extending

from 670 m.c. at the centre of the field to 6500 m.c. at the source, they usually moved away from the light before tracing a circular path either to the right or left, which brought them into a position facing up the gradient. This initial turning movement was a prelude to the mites tracing paths straight towards the source, but with a tendency to curve to one side of the beam (see Fig. 1 B). The movements of mites exposed to a weaker beam, with a light gradient extending from 270 to 2600 m.c., did not show the same features. The initial turning, induced by the strong beam, was not evident, and the tracks towards the source were decidedly wavy, and only straightened near the source (see Fig. 1 A). Occasionally a mite moved away from the light and when transferred back to the centre of the field it repeated

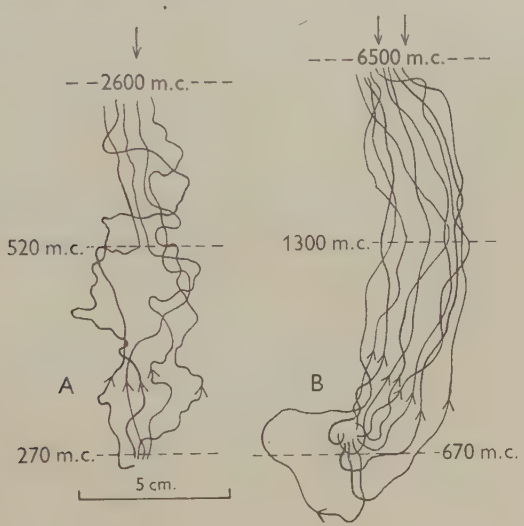


Fig. 1. Tracks of harvest mites in a horizontal beam of light. A, in a beam graded from 2600 to 270 m.c.; B, in a beam graded from 6500 to 670 m.c.

the negative response. Sometimes a photopositive mite when repeatedly brought back to the centre of the field orientated towards the light by taking almost the same path it had traced previously. Behaviour of this kind has probably been responsible for the suggestion that each animal inherits a specific response to an offered stimulus.

Two horizontal beams. In a two-light experiment with beams of equal intensity arranged to intersect at about 90° at the centre of the field, the mites were inclined to follow a path some distance along the bisector, before moving towards one or the other light source (see Fig. 2 B). When two unequal crossed beams were presented the mites moved directly up the gradient of the stronger beam (see Fig. 2 A).

To discover the ability of the mites to detect a stronger light intensity behind them, they were allowed to move up the gradient of a single beam until they had approached within about 6 cm. of the source (2600 m.c.), before an opposing light of 6500 m.c. at the source was switched on. The mites continued in the same direction for about a centimetre, stopped, made a complete turn, and then moved towards the stronger

light. Occasionally a mite displayed continuous turning movements on reaching the centre of the field (see Fig. 3).

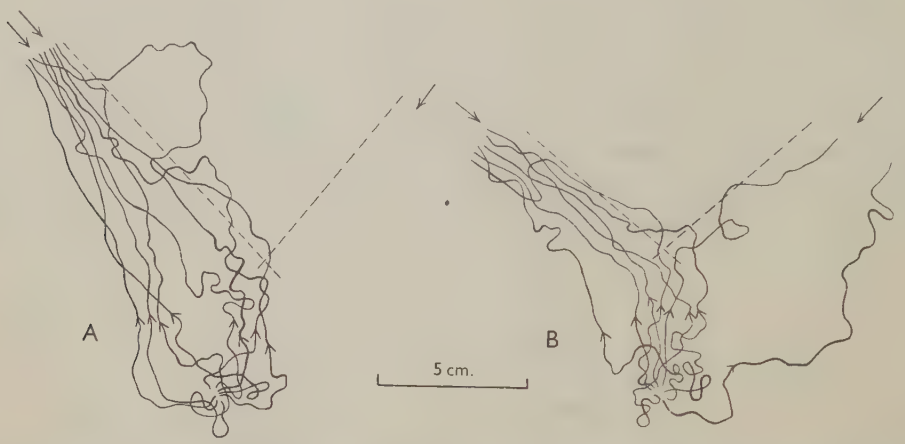


Fig. 2. Paths followed by harvest mites in two horizontal beams of light which intersect at right angles. A, in two unequal lights, one of 6500 m.c. (⇉), the other of 2600 m.c. (→), B, in two equal lights of 2600 m.c. (→).

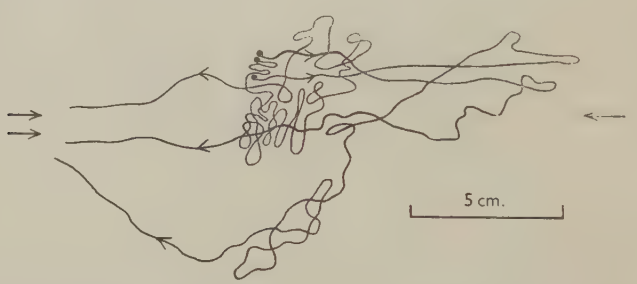


Fig. 3. Tracks of harvest mites between two unequal lights. The opposing light of 6500 m.c. (⇉) was switched on when the mites had moved within 2 or 3 cm. of a light of 2600 m.c. (→).

Response to a laterally presented beam

In this series of experiments the light source remained in the same position, whereas the filter-paper was turned about a centre point like a record on a gramophone disk. The mite was placed in the beam and allowed to move towards a light. The filter-paper and the mite were then turned clockwise through an angle of 90°. The light now fell laterally upon the mite so that one eyespot was more stimulated than the other. This asymmetry of stimulation caused the mite to re-orientate itself towards the light.

By repeatedly turning the filter-paper in this way the mite was induced to trace a rectangular path which brought it back to the starting point (see Fig. 4). However, individual mites showed variations in their reactions. The turning began either immediately or after a slight delay. Occasionally a mite after each turning movement traced a path directly towards the source of light. More often they moved towards it

at an angle of about 15°, but they corrected the deviation if they were allowed to continue to approach the light source.

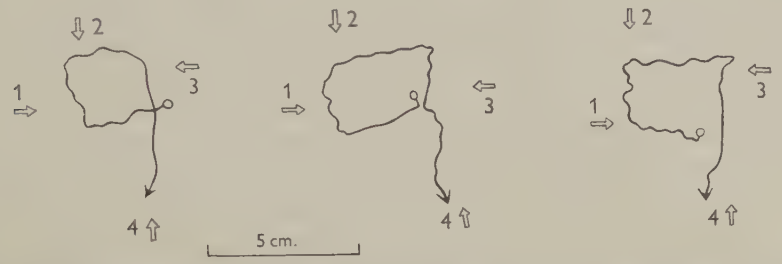


Fig. 4. Response to lateral illumination. The beginning of each track of the harvest mite is denoted by the circle, and the position of the light is indicated by the arrow numbered 1; thereafter the light was presented laterally.

Blinding experiments

Mites previously chilled to make them inactive were transferred to a moist filter-paper under a binocular microscope. Quick-drying black cellulose paint was placed, with the end of a single camel hair glued to a wooden handle, over the ocular area. Treatment was considered successful if the paint completely covered the area without interfering with any part of the body concerned in locomotion. The treated mite was then transferred to a glass cell and kept in complete darkness for an hour.

When mites blinded on one side were placed in a uniform light of 250 m.c. provided by an overhead lamp, they moved in the direction of the seeing eye, and made circus movements as they traced their somewhat random tracks towards the periphery of the experimental field (see Fig. 5B). Unblinded mites made more or less straight tracks from the centre to the periphery of the field.

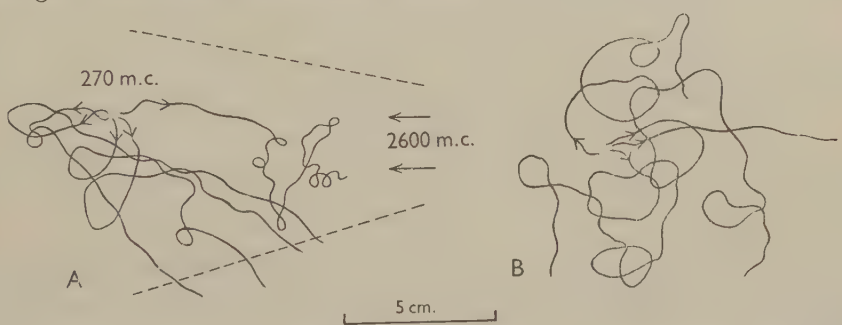


Fig. 5. Tracks of harvest mites with the left eye blinded. A, in a horizontal beam of light; B, when exposed to an overhead light.

When unilaterally blinded mites were exposed to a horizontal beam, they were inclined to make a gently curving circus movement before tracing a path diagonally across the beam and into the shaded part of the field. It was clear that the primary effect of the light was to stimulate the seeing eye, and increase the muscular tone of the uncovered side sufficiently to cause a deviation to one side as the mite approached the source (see Fig. 5A).

The response of totally blinded mites was also tested to discover whether or not a diffuse dermal light sense existed. In the way already described, mites with both eyes blinded were exposed to a horizontal beam of light, but, it being exceedingly difficult to accomplish the satisfactory blinding of both eyes of an individual mite, the number of tests was limited. The results of these tests (see Fig. 6) are not easy to interpret, but it is clear that the blinded mite completely loses the ability to move directly towards the light source. Apparent movement towards the light, when it occurred, could be accounted for by the possibility of light entering the thin cuticle, and reaching the eyes from below (Oehring, 1934, after Wigglesworth, 1939). This is certainly a possibility because mites, contained in a glass jar exposed to light, will take up a position nearest to the source, although this entails the ventral side of their bodies facing the light (see p. 481). The same response is equally well demonstrated by their response to the horizontal window of a light-trap, where the mites, walking upside down upon the under-surface of the window, remain in the incident light (Jones, 1950*a*).



Fig. 6. Tracks of harvest mites with both eyes blinded, in a horizontal beam of light from a source of 2600 m.c. (→).

Reactions to light intensity

The experiments were designed to discover whether the mites showed a selective activity towards light intensities under conditions in which the source of light was not apparent.

A glass tube, 27 cm. in length and 0·6 cm. in diameter, closed at each end by a ground-glass stopper, was marked off into three equal lengths A, B and C. Section A was blackened with a deposit of Sudan black. The middle section B was shaded by an opaque screen to give a light intensity of about 300 m.c., whilst the light intensity of section C, exposed to the northern light of the window, was about 500 m.c. Fifty mites were placed in the middle of the tube for each test. The mites scattered along the tube, and whereas they avoided the darkened section there was no indication that they showed a preference for the window light. The mites were

continually active and moved to and fro indiscriminately between sections B and C. The movements of individual mites are shown in Fig. 7.

To accentuate the difference between the light intensities the glass tube was placed in direct sunlight. A moist pad of cotton-wool was inserted into the tube near the closed end of the darkened section to provide a favourable air humidity inside the tube. The mites still avoided the darkened section, but occasionally they wandered into it for a brief period. Rough positional counts, made every 15 min., of mites in the shaded section B and the sunlit section C showed that about two-thirds of the number of mites tended to remain in the sunlit portion. Tests carried out with individual mites showed that frequently the mite, when placed between sections B

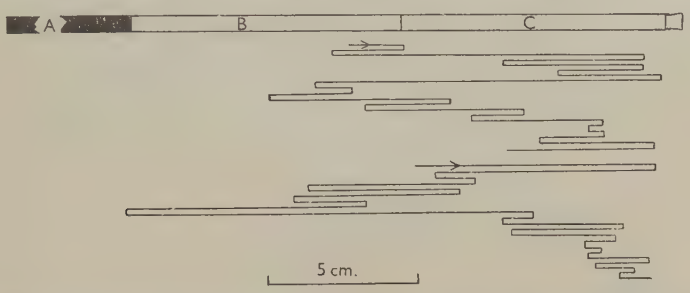


Fig. 7. Movements of harvest mites inside a horizontal glass tube exposed to different light intensities. A, darkened; B, shaded (300 m.c.); C, exposed to a light from a window facing north (500 m.c.).

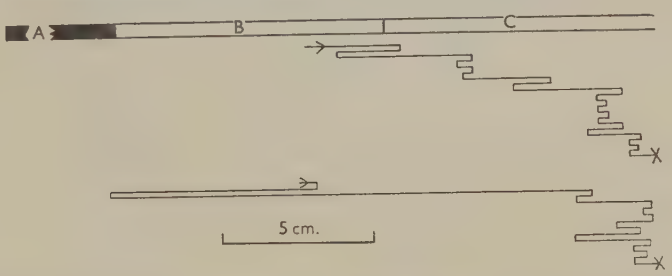


Fig. 8. Movements of harvest mites inside a horizontal glass tube of which section C is exposed to bright sunlight and section B is shaded with an opaque glass screen.

and C, first crawled away from the more intense light. Occasionally it entered the darkened section for a brief period before making very devious tracks towards the sunlit portion of the tube. But on having entered the sunlit portion the mite remained there actively crawling around the inside of the tube (see Fig. 8).

Such experiments, in which a portion of the tube containing mites was exposed to direct sunlight, did not allow for the possible influence of temperature and humidity upon the observed distribution of the mites. Extremes of light intensity were therefore offered to mites in a tube in which temperature and humidity variations were eliminated. The tube was marked off this time into four sections, A, B, C and D. Section A was darkened; B (300 m.c.) was shaded by an opaque screen; C (500 m.c.) was open to the window light; and section D (1000 m.c.) was exposed to a water-

cooled beam of light (see Fig. 10). The beam was screened from the other sections of the tube. The air temperature in each marked off portion of the tube was 19° C.

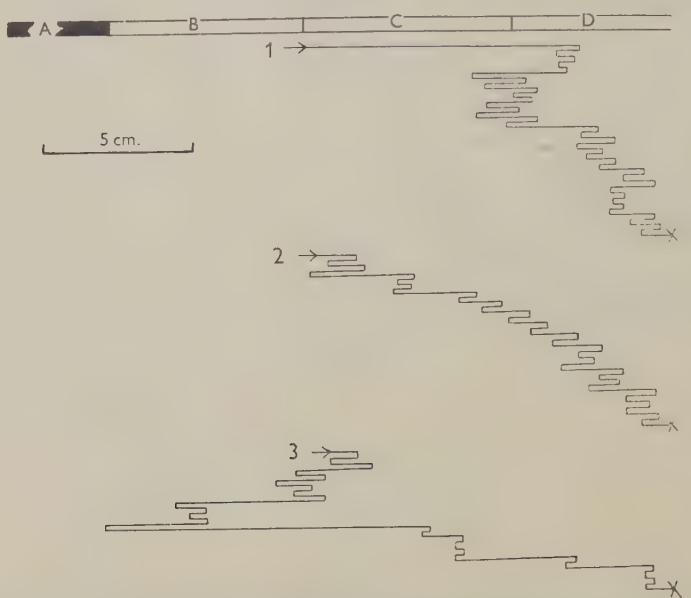


Fig. 9. Tracks of harvest mites inside a horizontal glass tube divided into four sections of different light intensities. A, darkened; B, shaded (300 m.c.); C, exposed to a window facing north (500 m.c.); D, exposed to artificial light of 1000 m.c.

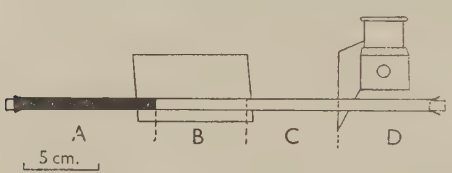


Fig. 10.

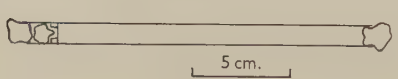


Fig. 11.

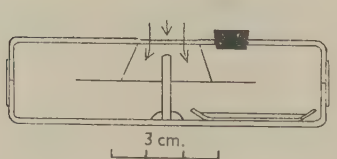


Fig. 12.

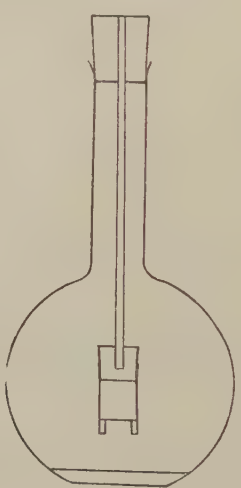


Fig. 13.

Figs. 10–13. For explanation see text.

Tests were carried out with individual mites. In most of them the mite moved straight towards section D and remained there. When the ground-glass stopper was removed at this end of the tube the mite displayed a well-defined preference for

crawling actively around the rim of the open end. The mite was usually removed after it had crawled on the rim for 20 min. or more (see Fig. 9). This response to the highest intensity of light indicated that the response to sunlight was a true response to light and not to heat. The avoiding reaction to darkness was the natural outcome of the photopositive mite orientating itself towards the lighter part of the field.

When mites are transferred to a collecting jar exposed (either in the field or in the laboratory) to daylight they become aroused and display an immediate upward-climbing reaction. They aggregate round the base of the glass stopper and penetrate any space between the stopper and the neck of the jar. If the stopper is removed they will crawl round the rim of the neck, but they are just as liable to climb directly over the rim, down the outside of the jar and to the substratum. The upward-climbing movement was not the result of an avoiding reaction to condensed moisture from moist filter-paper at the bottom of the jar, because the neck was kept moist with a wet camel-hair brush each time a fresh batch of mites was introduced. The behaviour suggested either a response by the photopositive mite to sunlight or negative geotaxis.

Response to shading

In the field it was not difficult to obtain from the mites a very definite questing response to a shadow suddenly passed over them. It was best elicited from a quiescent cluster of mites exposed to sunlight. When the hand was passed rapidly over a cluster of mites they displayed immediate and very definite reflex actions. The cluster was transformed into a mass of waving legs. The typical reaction of an individual mite was to direct the anterior part of the body upward and hold the forelegs out in front

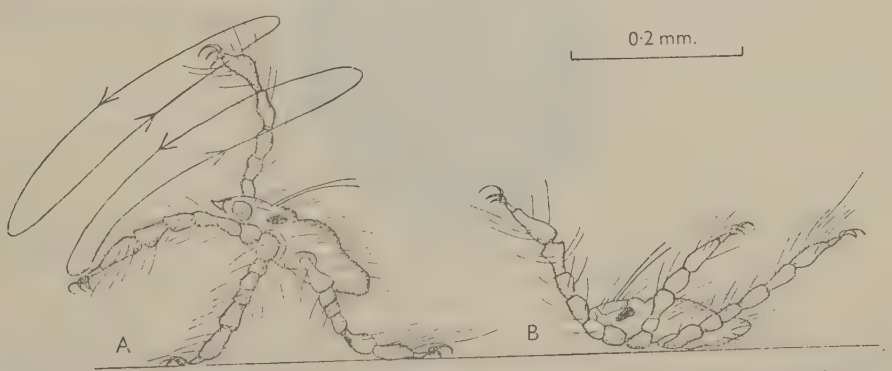


Fig. 14. Questing response (shadow reflex) of harvest mites. A, the waving forelegs; B, the body lowered and the legs directed upward.

of the body. The forelegs beat the air with alternate up and down movements as if the mite was trying to reach something immediately above. The frantic beating of the forelegs subsequently stopped and the cluster broke up, with the mites moving away from the site. A shadow passed over the crawling mites a sceond time stopped them in their tracks and induced the typical questing response. When a cluster of mites was dispersed by passing a shadow only once or twice it was common for the mites to reform into a cluster provided there was no further interference. If the

shadow stimulus was produced repeatedly the questing response gradually weakened as the aroused mites wandered in all directions away from the original site.

This response to a sudden decrease in the light intensity was observed in more detail under the microscope in the laboratory. When a shadow, thrown by a needle, was passed over them they immediately stopped in their tracks and the forelegs beat the air, whilst the body of the mite was supported by the mid- and hind-legs (see Fig. 14A). Frequently the body was lowered suddenly to the substratum, whilst all the legs were extended upward (see Fig. 14B). In the same way the shadow of a passing host would equally induce the mites to quest, a condition of preparedness, which would greatly improve their chances of being picked up.

Response to reflected light

Method. The experimental field consisted of an arena 14 cm. in diameter with a wall 6 cm. high. The arena was constructed by placing a collar of black paper upon a sheet of black paper which was the floor of the experimental field. A piece of white paper, 6 cm. square, served as a white screen against the black wall of the arena. An overhead lamp illuminated the arena with a weak but uniform light of 50 m.c.

Results. At first the mites usually turned away from the white screen before they moved towards it, but in all cases the screen was eventually reached. The tracks were



Fig. 15. Tracts of harvest mites towards a white screen presented in a black arena exposed to an overhead light.

very wavy, and those which were inclined to lead towards the side of the screen curved towards it as the distance shortened (see Fig. 15). None of the paths taken suggested that the mites reached the screen by a series of avoiding reactions to the dark surface of the arena, so that this response to a white surface suggests that it is one towards reflected light, an orientation described in many insects (Fraenkel & Gunn, 1940).

REACTIONS TO TEMPERATURE

Temperature is an important factor to insect parasites of warm-blooded animals, and is one of the stimuli guiding the insect to its host. Wigglesworth & Gillett (1934) found that *Rhodnius prolixus*, the large South American blood-sucking bug, moved

towards a test-tube of warm water, and that the receptors for detecting warmth were located in the antennae. Rivnay (1932) showed that the bed-bug is thermotactic. Mosquitoes and some lice are also stimulated to bite by the heat of the body. Totze (1933) mentioned, and Lees (1948) later confirmed, that the tick, *Ixodes ricinus*, will move towards a warm tube. The common occurrence of harvest mites on warm-blooded hosts suggested the possibility of temperature being a guiding stimulus.

Response to a source of heat

Method. The purpose of the experiments was to learn whether a concentric temperature gradient set up around a heated glass tube could guide the mite towards the tube itself.

A flat-bottomed glass tube, 2 cm. in diameter, was connected by a capillary tube arrangement to an adjacent large vessel containing warmed water. The warm water circulated through the tube at a controlled temperature, and ran off through an outlet capillary tube. A thermometer was also inserted into the tube to take temperature readings. The tube itself was placed in the centre of a filter paper, the mites (previously kept at a temperature of 18° C.) were released at varying distances from it. The temperature of the laboratory whilst the tests were being carried out was 18–19° C.

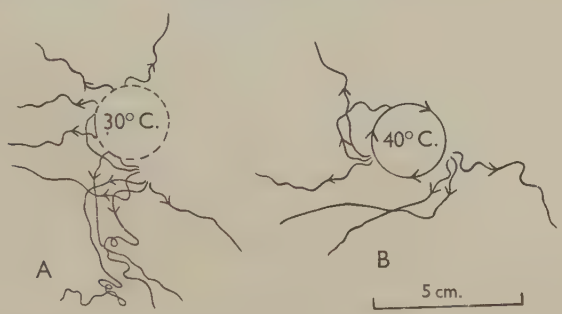


Fig. 16. Responses of harvest mites to a warmed glass tube. A, tracks around a glass tube warmed to 30° C.; B, tracks around a glass tube warmed to 40° C., with a circular track representing movement around the base.

Results. Mites released at distances of 1 and 0·5 cm. from warm tubes with temperatures of 25, 30, 35, 40 and 45° C. showed no response. Occasionally individual mites in their wanderings from the point of release moved towards the tube and touched it. At temperatures of 25–30° C. the mites which had moved against the heated tube showed no positive response and continued their wanderings away from the tube towards the edge of the filter-paper (see Fig. 16A). At temperatures of 35–40° C. mites which had accidentally touched the tube displayed a well-defined response. For a period varying around 3 min. they crawled continually around the base of the heated tube before climbing upon it (see Fig. 16B). Mites placed against the base of the tube at temperatures between 35 and 40° C. invariably displayed this definite reaction of excitedly crawling around the base of the tube. They were not

repelled by a temperature of 45° C., but they crawled around the base of the tube until the temperature had fallen to about 40° C. before climbing upon it.

Since the temperature from which the mites were taken was about 19° C. and the temperature to which they reacted was 35–45° C., the indication was that a temperature difference of about 15° C. was required to induce a positive response. It was expressed only as an accelerated rate of locomotion; on no occasion did the forelegs quest as in the shadow reflex. So the forelegs do not act as thermo-receptors like the forelegs of the tick or the antennae of some insects.

Reactions to a linear temperature gradient

In investigating the reactions of small animals to a linear temperature gradient, MacLeod (1935) used a copper strip cooled at one end and warmed at the other. Wigglesworth (1941) used an apparatus consisting essentially of a zinc trough embedded deeply in a large trough containing sand. The zinc trough was cooled with ice blocks at one end and warmed by means of a series of graded Bunsen flames. In the present work it was convenient to use a tubular linear temperature gradient apparatus.

A glass chamber, 27 cm. long and 1 cm. in diameter, was closed at one end with a rubber stopper, whilst a rubber stopper carrying a short length of narrow glass tubing was fitted into the other end. A thermometer was inserted into the chamber through the glass tube. The bulb of the thermometer could then be moved to any required region of the chamber in order to record the air temperature.

The chamber was placed parallel to a window facing north. The closed end, for a distance of 4 cm., was embedded in ice, the opposite end rested upon a trough filled with sand gently heated by a Bunsen flame. A temperature gradient extended from 7 to 40° C., ±2° C., was maintained. A piece of filter-paper carrying about fifty mites was placed in the middle of the chamber. The mites had been previously kept at 18° C. They quickly dispersed inside the chamber, but displayed avoiding reactions as they approached the lower and higher temperatures of the gradient. In the six experiments rough positional counts were made at intervals. The results indicated that there was not a well-defined preferred zone, but most of the mites were aggregated between temperatures of 15 and 26° C. at any one time (see Table 1).

Table 1. *Average distribution of fifty harvest mites when offered a linear temperature gradient, graded from 7 to 40° C., ±2° C., inside a glass tube 27 cm. long and 1 cm. in diameter.*

Temp. (° C.)	7–10	10–11	11–15	16–20	20–25	25–30	30–40
Average no. of mites	2	4	6	26	11	1	0

Reactions to a concentric temperature gradient

The purpose of the tests was primarily to discover the nature of the movements of the mites away from the lower and higher temperatures of a gradient, because it was not easy to interpret these movements inside a glass tube.

A ground-glass plate, 30 cm. in diameter, was placed upon a peripheral circle of ice blocks. A blackened 25 W. bulb was placed below the centre of the under-surface of the glass plate. The ice reduced the temperature at the edge to about 7° C., whilst a temperature of about 40° C. was maintained at the centre. The temperatures were measured approximately by placing the bulb of a thermometer against the glass plate.

The mites were released at the centre of the experimental field. They moved from the centre towards the edge of the field. On approaching the cooler part they displayed avoiding reactions. Similar reactions were observed in the central warmer region. It was noticeable that as a mite moved into the cooler part the rate of locomotion decreased, but it was never immobilized or failed to turn round and walk in the opposite direction towards the warmer regions. On approaching the warmer parts the mite displayed a detectable increase of pace before turning round and crawling back down the gradient. The turning away from the cooler or warmer parts of the gradient was usually gradual and seldom abrupt. Moreover, the recorded paths of the mites clearly showed that as the mites moved around the circular field they did so within a well-defined zone owing to a series of avoiding reactions to the lower and higher temperatures of the concentric gradient (see Fig. 17).



Fig. 17. Response of harvest mites to a concentric temperature gradient.

RESPONSE TO CHEMICAL STIMULATION

Henschel (1929) investigating the olfactory sense of *Tyrolichus casei*, the cheese mite, showed that it reacted positively to putrefying protein and one of its constituents skatol, provided the concentration was not too high. The cheese mites reached baits such as meat juice and squashed caterpillars by very devious paths up the gradient of intensity. When the tick *Ixodes ricinus* is stimulated chemically it starts waving its front legs (Totze, 1933). The front legs bear Haller's organs which are the chemoreceptors. Totze (1933) also showed that the tick was capable of picking out an optimum zone in a gradient of butyric acid; Solomon (1937) found that various substances were repugnant to *Halotydeus destructor*, the red-legged earth mite. It is

difficult to decide whether a mite has an olfactory sense, for although many workers assume that blood-sucking insects have a sense of smell and are attracted to the host by the odour emanating from the body, difficulty arises in discriminating between an olfactory sense and a sense in the nature of taste.

The very excitable and purposeful movements of harvest mites crawling on the hairs or naked skin of a host suggested that odour was a possible stimulant. Their predilection for certain habitats on the host itself further suggested that odour might exert a guiding influence. Host tissues and secretions as well as various volatile substances were therefore tested on the mites.

Reactions to volatile substances

Di-methyl-phthalate and di-butyl-phthalate were most successfully used during the military campaigns in the Far East for repelling Trombiculid mites. Certain other substances commonly used in the laboratory were found to be repugnant to the mites, and these were used in tests designed to assess their sensory perception of chemical stimulation.

Fifty mites were transferred to a glass tube 15 cm. long and 1 cm. in diameter. A small pad of cotton-wool was moistened with the test substance and placed inside the tube near one end, being separated from the mites by a fine lawn screen supported by a rubber ring. The open ends of the tube were plugged with cotton-wool (see Fig. 11). Light was directed upon the end containing the test substance so that the mites, being photopositive, were induced to move towards it.

It was found that the mites displayed avoiding reactions on approaching within 0·5 cm. of phenol, methyl-phthalate, dilute ammonia, xylene and a 3% solution of glacial acetic acid. Toluene appeared to have a stronger effect; the mites were repelled at a distance of 1·5 cm. A mixture of 3 vol. of amyl acetate and 97 vol. of water was even more repellent, for the mites reacted to the vapour at a distance of 5 cm. Mites approaching within this distance were narcotized by the vapour. Indifference was shown to glycerine and lactic acid.

The mites did not wave the forelegs in the manner described by Totze (1933) in his observations on the reaction of ticks to chemical stimulation. Since the mites were only able to detect the vapour at very short distance it must be assumed that their sensory perception of chemo-stimulation is not well developed and that high concentrations of vapour are necessary to induce avoiding reactions. Although the mite possesses sense organs of the chemo-receptor type (see p. 485) the entry of the noxious vapour into the tracheal system which opens near the chelicerae may also have played a part in the perception of the volatile substances tested.

Reactions to host tissues and secretions

The host tissue or secretion to be tested as an attractant was placed in the centre of a filter-paper. The mites were released at various distances and from different positions around the test substance. The tests were conducted at a constant temperature of 25° C.

Skin. Mites released at distances of 0·5 and 1 cm. from a piece of skin of a freshly killed mouse were indifferent to its presence. Their tracks showed no relation to the piece of skin (see Fig. 18 A).

Liver. The odour of a piece of fresh mouse liver proved equally negative (see Fig. 18 B).

Sebum and cerumen. The odour of hair emanates from the oil secreted by the sebaceous glands which are located around the hair roots. The odour of mouse, rabbit and human hair were tested on the mites but they showed no response (see Fig. 18 C). Complete indifference was also shown towards the odour of human and rabbit cerumen—the waxy secretion of the external meatus—which contains a high percentage of sebum (see Fig. 18 E). Thus cerumen would not appear to play a part in attracting the mites into the ears, which are their characteristic habitats on rabbits.

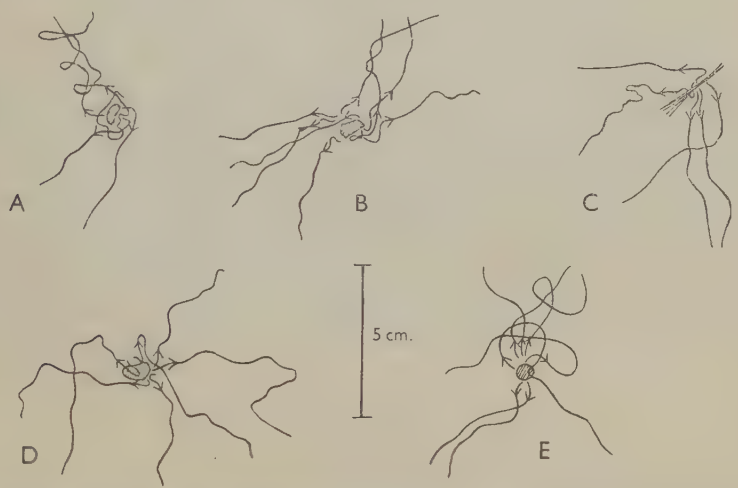


Fig. 18. Tracks of harvest mites released at varying distances from host tissues and secretions at 25° C. A, skin; B, liver; C, hair; D, perspiration; E, waxy secretion of external meatus.

Perspiration. A small pad of cotton-wool moistened with perspiration taken from the body of a dark-skinned person was placed on a filter-paper. Mites released at distances of 0·5 and 0·25 cm. showed complete indifference, but those which made contact or near contact with the test substance displayed avoiding reactions (see Fig. 18 D). Of 100 mites, placed five at a time, alongside the test substance, forty-six exhibited a negative response whilst the remainder were indifferent, although twelve mites crawled on to the cotton pad for a brief period. The presence of decomposition products in the perspiration were probably responsible for repelling the mites. Totze (1933) showed that a high concentration of butyric acid, which is one of these products, repelled ticks. A pad of cotton-wool moistened in this substance was repugnant to the mites.

The results were interesting because they help to explain why some people are not troubled by the attentions of harvest mites. Dark-skinned people, who tend to perspire more than fair-skinned people, are less severely attacked by the mites.

Differences in the composition of the perspiration between one person and another are probably partly responsible for differences in the extent of an attack by the mites, because a few tests indicated that the perspiration of one person was found to be more repugnant than that of another person. For example, only twelve out of 100 mites, placed in batches of five alongside a pad of cotton-wool moistened with perspiration taken from a fair-skinned person, displayed a well-defined avoiding reaction. However, the questions of differences in the degree of repulsion of perspiration of various hosts and of whether dark-skinned people are more immune than fair-skinned people do not come within the scope of the present paper.

REACTIONS TO A YOUNG LIVE MOUSE

A young live mouse was chosen as the most suitable experimental host and placed in the centre of a filter-paper. Mites released at distances of 0·5, 1, 2 and 3 cm. showed complete indifference to its presence. Occasionally individual mites moved towards the mouse and made devious paths under the legs and tail. Even then they usually continued their tracks away from the mouse and towards the edge of the filter-paper. Mites placed in close proximity with the body of the mouse also showed indifference. But on climbing over the mouse and making contact with the skin the mite then displayed the striking behaviour so characteristic of a mite crawling on the hand (see p. 481). It continued crawling at an accelerated pace upon the surface of the body for 30 min. or more (see Fig. 19). On reaching the distal parts of the limbs or tail it as a rule showed no inclination to crawl off to the filter-paper but instead clung tenaciously to the skin and retraced its tracks towards the body.

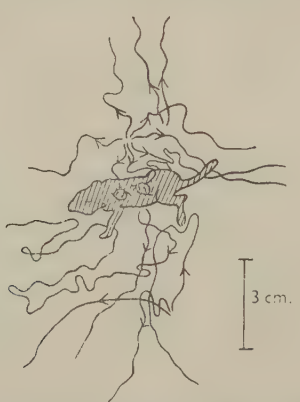


Fig. 19. Tracks of harvest mites released at varying distances from a young live mouse. The tracks, marked with a circle, led the mites to the body of the mouse, where they remained crawling incessantly for a prolonged period.

It was evident from this behaviour that some stimulus other than touch was responsible for causing them to crawl incessantly over the body of the mouse. However, in the odd case when a mite crawled off, it never turned round in an attempt to locate the mouse but instead made tracks towards the edge of the filter-paper. Occasionally a mite climbed back to the mouse after accidentally remaking

contact with an outstretched limb. It then crawled incessantly at an accelerated rate over the body for a prolonged period. Mites previously kept on the body of the mouse for 30 min. were placed at appropriate distances from the host, but they, too, showed complete indifference unless they touched it accidentally and climbed upon its body.

It was evident that the mites were incapable of detecting the presence of a young live mouse even when they were almost touching it. But actual contact with the body of the mouse produced a response typified by an immediate increase of pace and a pronounced excitability. The body heat of the mouse, estimated at approximately 35° C., probably accounted for the well-defined accelerated pace and pronounced character of the response. The behaviour closely resembled that displayed by the mite crawling around the base of a glass tube warmed up to a temperature of 35–40° C.

RESPONSE TO HUMIDITY

It is well known that compared with work on the reactions of animals to temperature and light very few observations have been made on responses to humidity. In the natural environment the mites take shelter from excess free water after heavy rain. On wet days any shelter is taken advantage of in the micro-habitat, although most of the mites cannot avoid being washed into the soil. The fact that mites will survive for 4–5 days submerged in water suggests that heavy rains are not a serious handicap to them apart from restricting their movements. The importance of humidity as a limiting factor gave reason to believe that the mites were capable of perceiving variations of the moisture content in the air or soil (Jones, 1950*a*).

Reactions to atmospheric moisture

Method. For investigating the reaction to a humidity gradient a glass tube 15 cm. long and 1 cm. in diameter was used. A moist pad of cotton-wool at one end and calcium chloride at the other were separated from the introduced mites by plugs of cotton-wool. Mites previously exposed either for 24 hr. at 100% R.H. and room temperature or for 6 hr. at 50% R.H. and 24° C. were placed in the humidity gradient in the tube.

Five tests were made with batches of fifty mites taken from saturated air. The initial phase was characterized by the introduced mites moving actively in all parts of the tube, but whereas they displayed well-marked avoiding reactions to the moist end they climbed upon the cotton pad at the dry end and even pressed themselves between the cotton-wool and the glass tube. In one of the first tests the loose pieces of calcium chloride were not separated from the mite by a pad of cotton-wool. In this case the mites climbed upon the calcium chloride and even settled there. Avoidance of the moist end appeared to be responsible for most of the mites displaying an apparent preference for the dry end. Positional counts made at intervals during the first hour showed that most were recorded at the dry end (see Table 2). It was difficult to account for every mite because many had settled in the cotton-wool. After the first hour a few had settled on the moist pad, but most of them were still

active and were wandering to and from the moist end. After 3 hr. there were still a greater number of mites at the dry part, but the number of mites aggregated upon the moist cotton-wool had also increased. After 6 hr. clusters of mites were forming on the moist cotton-wool and few mites were left upon the dry pad. After 24 hr. there was a complete reversal in distribution, practically all the mites having settled at the moist end (see Table 2), where many of them were by this time trapped in condensed moisture. Occasionally mites were trapped after penetrating the cotton pad at the dry end, and after a prolonged period of exposure they died through desiccation. Mites introduced into a tube closed at each end with a moist pad were at first very active and avoided both ends, but after 3 hr. they settled and distributed themselves more or less uniformly (see Table 2). The settled mites showed a tendency to cluster on or near the moist pads, but this could have been accounted for by their becoming trapped in condensed moisture.

Table 2. *Distribution of fifty harvest mites, previously exposed for 24 hr. at* 100% R.H., *when offered alternate high and low humidities in a glass tube*

Time	Alternative humidities				Control			
	100% R.H.		25% R.H.		100% R.H.		100% R.H.	
hr. min.	Total	Active	Total	Active	Total	Active	Total	Active
30	11	11	39	35	20	10	30	14
1 0	12	10	38	34	18	12	32	16
3 0	18	11	32	29	22	21	28	26
6 0	38	14	12	9	—	—	—	—
24 0	46	2	4	1	—	—	—	—

Mites previously desiccated for 6 hr. at 50% R.H., 25° C. behaved in a humidity gradient differently from those taken from saturated air. They were very active after release in the tube, but avoidance of the moist end was not so evident. After 1 hr. the mites, now much less active, were more or less uniformly distributed along the gradient. Only a few mites had climbed upon the pad at the dry end. After 3 hr. most of the mites had aggregated at the moist end and clusters had formed on the moist pad (see Table 3).

Observations on the behaviour of normal and desiccated mites in a humidity gradient suggested that the initial distribution of normal mites in the dry region is

Table 3. *Distribution of fifty harvest mites, previously exposed for 6 hr. at* 50% R.H., 24° C., *when offered alternative high and low humidities in a glass tube (see text)*

Time	Alternative humidities			
	100% R.H.		25% R.H.	
hr. min.	Total	Active	Total	Active
15	26	26	24	24
30	27	25	23	23
1 0	28	22	22	18
2 0	36	18	14	10
3 0	38	12	12	9

the result of an avoiding reaction to saturated air. Since desiccated mites did not show this well-marked avoidance of the moist end, the expression of the avoiding reaction would appear to depend on the condition of humidity to which the mites had previously been exposed.

The eventual aggregation, of normal mites after 24 hr. and desiccated mites after 3 hr., at the moist end is due to a forced movement away from the dry region owing to water loss by evaporation through the cuticle. This depletion of the water balance stimulates the mites into activity, the random movements leading them into the moist region where movement is arrested whilst the water balance is restored. Hygrokinesis was more strongly displayed by the desiccated mites, which settled more quickly in the moist region, than by those previously exposed to saturated air. This behaviour of the mites towards humidity closely resembles that of the tick (Lees, 1948) and the spider beetle, *Ptinus tectus* (Bentley, 1944).

Reaction to free water

A circle of filter-paper was wetted thoroughly at the periphery with a wet camel-hair brush. The water film 'crept' from the edge towards the centre. Mites were then released at the centre of the filter-paper, and they displayed definite avoiding reactions to the surface film of the barrier ring of water (see Fig. 20).



Fig. 20. Response of harvest mites to free water. Barrier ring of water is represented by the area between the dotted line and the outer circle.

When the wetting operation was stopped and evaporation of the water from the filter-paper reduced the amount of water in the outer circular area, the mites made devious paths across the moist region towards the edge of the filter-paper.

Humidity and survival

Several workers, including André (1928) and Keay (1937), have emphasized the importance of a high humidity for the successful rearing of the fed larva to the nymphal stage. Results of a previous investigation (Jones, 1950*a*) suggested a relation-

ship between a high relative humidity and the distribution of the mites, but the resistance of the mites to dry conditions liable to be encountered in the natural environment remained obscure. Mites were therefore exposed to various air humidities to discover the most favourable one for survival.

Method. The mites were exposed to various humidities in a constant-temperature room at 24° C.

To produce the required humidity appropriate strengths of sulphuric acid were introduced into a series of 2 l. flasks. Six flasks were prepared with the following range of humidities: 0, 10, 25, 50, 90, and 100%. Each flask was closed with a rubber stopper which carried a glass rod at the end of which was suspended a glass tube $3\frac{1}{2}$ cm. long and $2\frac{1}{2}$ cm. in diameter. The glass tube was closed above by a rubber stopper and below with a piece of lawn held tightly against the side of the tube by a rubber ring (see Fig. 13). The chamber successfully retained the mites. The flasks were kept in the constant-temperature room for 2 days to allow the humidity in each flask to attain equilibrium before the mites were introduced into the chamber.

Preliminary tests showed that hourly examinations were sufficient, because even at 0% R.H. the mites remained alive for 6 hr. Twenty mites previously exposed to saturated air were introduced into each chamber of the six flasks in six successive humidity tests. When a close examination of the state of the mites was required, the glass tube was quickly removed from the flask and placed under a binocular microscope for as brief a period as possible, during which time the flask was stoppered.

The mites were at first very active at all humidities. After 3 hr. the movement of most of the mites in saturated air was arrested, but at the other humidities the mites were still active. At the lower humidities, 10–50% R.H., they were continually active until the depletion of water through evaporation proved fatal. Desiccation reduced the body size of the mites considerably at the end of 6 hr. at 0% R.H. The sustained activity was a response associated with the depletion of the water content. This activity was quite pronounced at 90% R.H., and the survival time was appreciably less than that recorded for mites in saturated air. The average survival times at different humidities are shown in Table 4. It was not difficult to recognize the shrivelled appearance of a desiccated mite when assessing the mortality.

Table 4. *Average time required to produce* 100% *mortality of harvest mites when exposed to varying degrees of relative humidity at* 24° C.

Percentage relative humidity	0	10	25	50	90	100
Average time in hours required to produce 100% mortality	6	6	10	19	33	70

An atmosphere saturated with moisture was clearly the most favourable for survival. The appreciable resistance time of 6 hr. to 0 and 10% R.H. may have been due to a use of the water phase of the tracheal system. The results of Davies (1928) in his investigations on the effect of various humidities upon the survival of Collembola revealed that tracheate species survived longer than atracheate ones.

REACTIONS TO TACTILE STIMULI

When a quiescent cluster of mites was touched lightly with a dry camel-hair brush the mites immediately quested very actively and showed a remarkable ability for climbing upon the hairs, from which it is difficult to remove them as they cling on tenaciously. The aroused mites will climb upon the wooden handle, and if given the opportunity will transfer themselves in a purposeful way to the fingers. If the mite is retained on the handle or brush part for a prolonged period, it stops more frequently and gradually loses its initial excitability. The behaviour on the warm skin is quite different; with tenacity and at relatively great pace the mite climbs straight from the hand to the arm whether it is held upward or downward. This response to contact with the warm skin is very striking. The photopositive response is certainly obscured by it, since the mite will move from the exposed hand to the darkness of the covered parts of the body--a curious reversal in behaviour towards light in an animal whose unfed state has not changed.

When mites are placed upon the body of a rabbit or mouse they move with remarkable agility from one part of the body to another, and crawl at random for some considerable time before they attach themselves to the skin. During this preattachment phase they suffer a high mortality rate owing to the attentions of the host. Observations of their movements upon the bodies of young mice suggested that a tactile sense was primarily responsible for the clusters upon typical sites of a host. It is significant that inside the ears or around the anus or between the digits are favoured habitats, and one would expect this because by their very nature as 'niches' they are most likely to induce contact between the mites.

Mites often aggregate upon the corners of wooden planks or upon fallen branches. When the wooden plank or branch was lightly tapped the resulting vibrations induced an immediate questing response.

CLUSTERING

The response to contact with each other's bodies is very noticeable in mites living on the soil, and reference has been made in an earlier paper (Jones, 1950*a*) to the part this tactile response plays in the gregarious habit of forming clusters. But the phenomenon of clustering has been given further and separate attention in this paper in its relation to the influence of light, humidity and gravity.

The influence of light

About fifty mites were introduced into a closed tube partly lined with moist filter-paper. Occasionally a few mites were trapped in droplets of water. In the dark room the light of an overhead lamp induced the mites to climb upwards. After a prolonged period of 3 hr. or longer they formed a cluster at the top of the tube, behaviour resembling that of mites in a collecting jar exposed to daylight. A lamp placed at the side or below the glass tube induced the mites to form a cluster at a point nearest to the source of light. The upward-climbing movement in such cases

was not apparent. The results suggested the upward-climbing action to be a response to light intensity. A negative geotactic response, if it does exist, is most certainly weaker than the photopositive response.

The influence of humidity

A wooden rod, supporting at half-way a circle of black filter-paper, 5 cm. in diameter, was glued at one end to the centre of the inside of a blackened Petri dish. Another equal-sized Petri dish, blackened except for a central circular area of about 2 cm. in diameter, was inverted and placed upon the other so that the untreated area was directly above the tip of the vertical wooden rod. They were held together with a strip of adhesive tape. Hence light entered the chamber through the circular window only, and it was conceivable that mites placed upon the suspended filter-paper within the chamber would respond to the light by climbing up the rod. The mites were introduced through a hole in the top Petri dish. The necessary strength of acid was introduced into the chamber to give the required humidity. The arrangement is shown in Fig. 12.

Fifty mites were used for each test, which lasted for at least 8 hr. and if necessary until the following day. At 10 and 50% R.H. the mites were very active and climbed continuously up and down the rod. At 90% R.H. they remained longer at the tip, but no clusters were formed. At 95% R.H. and in saturated air mites aggregated upon the tip and persistent clusters were formed at both humidities.

The influence of gravity

Several workers, including Hindle & Merriman (1912), MacLeod (1935) and Lees (1948), have obtained varying results in testing the response of ticks to gravity. Lees (1948) concluded that ticks placed on a vertical rod displayed a form of negative geotaxis, although occasionally they walked downwards for considerable distances before turning, but if they had recently experienced arrival at the tip of the vertical rod, the turning upwards was enhanced. Since MacLeod (1935) and Lees (1948) found that ticks were photonegative (at variance with the conclusion of Totze (1933), who found them to be photopositive), an explanation for the upward-climbing action of ticks had presumably to be sought in some form of gravity response.

In the case of harvest mites which are strongly photopositive, and sensitive to differences of light intensity, it was not easy to test their responses to gravity. On observing mites in the field, one would be inclined to suggest that some form of negative geotaxis influences the upward-climbing action. But the response to light could equally explain this movement. Therefore gravity tests on the mites had to be arranged with the lighting either evenly distributed in the vertical plane or entirely eliminated. Tests were arranged to determine the distribution of mites upon a vertical rod in uniform lighting at 20° C., 95% R.H.

The rod, 25 cm. long and 0·3 cm. in diameter, was a thin straight portion of a raspberry cane, which in the natural environment is included among the favoured sites for climbing. The rod was marked at intervals of 1 cm. Glass rods were unsuitable because they acquired a thin film of moisture which impeded the movement of

the mites. The rod was retained in position by two entomological pins fixed to an adjacent upright rod. Both rods were enclosed in a glass tube. The required humidity was obtained by lining the glass tube with filter-paper moistened with the appropriate salt solution over part of its surface. This arrangement (see Fig. 21) resembled that of Lees (1948).

The humidity of the air inside the glass tube was allowed to attain equilibrium before each test. Opaque screens were arranged around the apparatus to shield the glass tube from direct lighting, and the light intensity at the bottom and the top of the glass tube was about 300 m.c. The rod was withdrawn; the glass tube was closed with a temporary stopper, and approximately fifty mites were quickly placed upon the rod at the half-way mark, before it was replaced.

Positional counts were then made at suitable intervals, but the total number varied at different intervals, either because some escaped notice, or others had found their way across one of the pins to the supporting rod. Owing to this loss of mites, the total number at the end of a period of about 30 min. was invariably reduced. At the high humidity, one would expect that if some form of negative geotaxis existed, the mites would climb to the tip of the rod, and on contacting each other would aggregate as a cluster. The average results of eight tests are shown in Table 5.

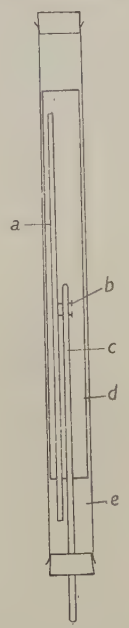


Fig. 21. Arrangement for testing the responses of harvest mites to gravity at a high humidity. *a*, testing rod; *b*, pin; *c*, supporting rod; *d*, filter-paper; *e*, glass tube.

Table 5. *Average positional counts, expressed as percentage, of harvest mites distributed upon a vertical rod exposed to uniform lighting at* 20° C., 95% R.H.

Section of vertical rod	Duration from start of experiment					
	5 min.	10 min.	15 min.	20 min.	30 min.	60 min.
Top 1	31·5	26·5	20·0	25·0	26·0	11·0
2	18·5	16·0	16·5	17·0	13·0	27·0
3	18·5	18·5	16·5	12·5	13·0	6·0
4	17·0	17·0	16·5	12·5	19·0	18·0
Bottom 5	14·5	22·0	30·0	33·0	29·0	38·0

The size and activity of the mites made it difficult to account for the total number during each count, but the figures, expressed as a percentage of each total count, indicate that when the mites are released upon the rod they are more inclined to climb up rather than down the vertical rod during the first 5 min. After this period the counts suggest that the mites distribute themselves fairly evenly along the rod. It was noticed, too, that some were inclined to walk round and round the rod within a restricted range of a few centimetres. On no occasion did the mites form a per-

manent cluster at the tip of the rod, although some tests were carried out in darkness for a period of 30 min. before the positions of the mites were examined.

The movements of individual mites were also followed. But the recorded tracks were so divergent in character that it was impossible to interpret them. The only feature which had some consistency was an initial tendency on the part of the mite to climb upwards rather than downwards or round and round one particular part of the vertical rod. One may conclude from the results of the tests that there is no specific indication of a negative geotaxis.

THE SENSE ORGANS

The external characters of systematic value for *Trombicula autumnalis* have been described by Hirst (1915). In doing so he naturally gave only brief descriptions of the external form of the ocular areas, various setae and their positions on the body, the scutum, and the appendages. Systematists have attached much diagnostic importance to the ciliation of the scutum, especially to the so-called pseudostigmatic setae which, in a somewhat misleading way, they have been inclined to designate specifically as 'sensilla', as if the considerable number of other setae present were of little significance as sensory end-organs.

According to the literature no study has been made of the structure and function of the various receptor organs of *T. autumnalis*, nor as far as I know has anything of the kind been attempted for any species of Trombiculid mite. Unfortunately, the minute size of the harvest mite is not conducive to carrying out elimination experiments, designed on the lines of those by Wigglesworth (1941), to locate the senses. Attempts that were made did not produce any satisfactory results, excepting those of the blinding experiments. But the analysis of the sensory perceptions of the mite justified a detailed examination of the sense organs, and since it had been possible to measure in some degree the intensities of the responses of the mite to various stimuli, it was desirable to try to identify the nature of the stimuli which the different receptor organs were capable of appreciating.

Sense organs in the legs

Plumose setae. These sensilla are confined mainly to the dorsal and lateral surfaces of the segments. Whether long or short they are slightly curved and taper to fine points from a base which measures about 2 μ in diameter.

In section this type of receptor is shown to arise from a chamber-like structure which penetrates the cuticle. In some sections there appeared to be ridges on the inside of the chamber (see Fig. 22 D). The sections were stained with haematoxylin, but whereas a sensory nerve in the thick-walled hair was well defined, it was difficult to make out a sensory process in the chamber which would link it with the nerve cells below the chamber itself. The nerve cells and their fibres associated with the sensilla were quite pronounced, but no structure resembling a trichogen or tormogen cell was visible in the harvest mite. The structure and the wide distribution of the plumose hairs are typical features of sensilla with a tactile function.

Plain setae. There are fewer of these sensilla and they are located only on the legs and palps. A conspicuous one, about 80 μ long, is present near the proximal end of the tarsus of the third leg. Their structure (see Fig. 22E), apart from the lack of barbs, is similar to that of the plumose setae, which suggests that they too are tactile sensilla.

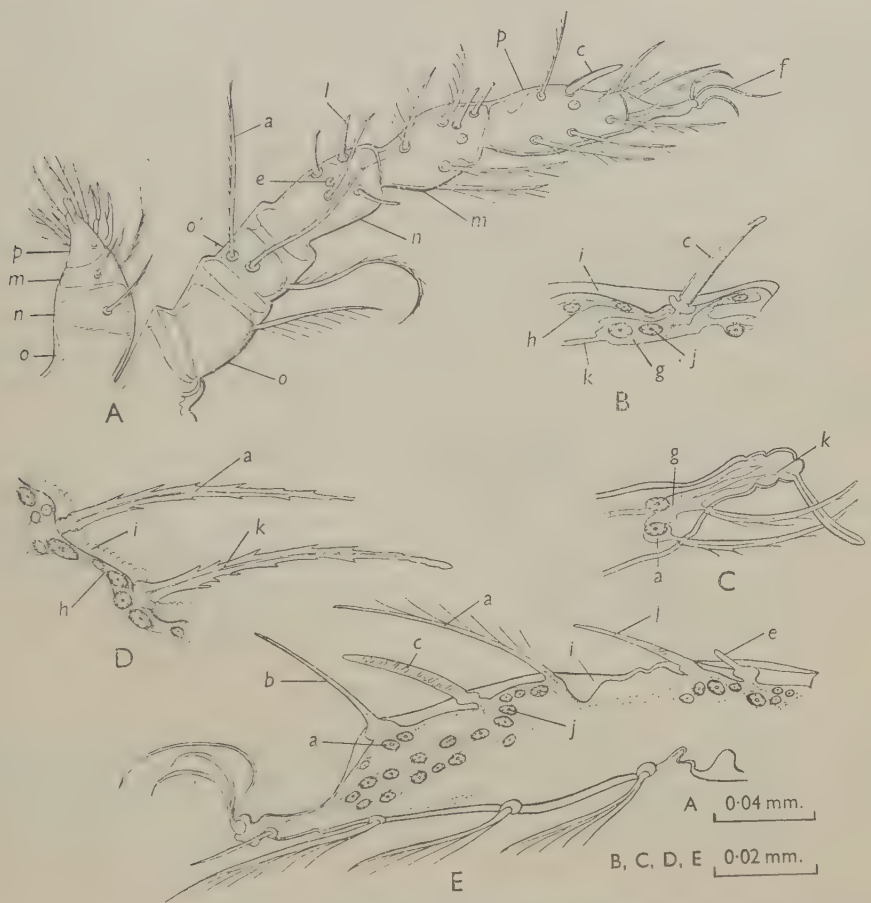


Fig. 22. A, dorsal view of right palp and first leg; B, detail of peg organ with round tip; C, detail of tactile sensillum and part of trifurcate claw; D, detail of plumose hair of body. E, detail of first leg. *a*, plumose hair; *b*, plain hair; *c*, peg-organ with round tip; *e*, small rod; *f*, claw; *g*, sense cell; *h*, hypodermis; *i*, cuticle; *j*, nuclei of sense cells; *k*, sensory nerve; *l*, peg organ with pointed tip; *m*, tibia; *n*, patella; *o*, basi-femur; *o'*, telo-femur; *p*, tarsus.

The peg organs. These organs are quite distinct in shape. There are two types, one with a round tip, the other with a pointed tip. A relatively stout slightly curved peg organ with a round tip is present on the tarsus of the first leg (see Fig. 22A). Those with pointed tips are more curved and slender.

In section the peg organ is shown to be thin walled (see Fig. 22B), with a constriction at the base, so that a narrow aperture connects the cavity of the peg with that of the basal chamber which penetrates the cuticle. Below the chamber are sense cells

forming a nerve strand that can be traced to the chamber. The cavity of both chamber and peg contained a clear fluid-like substance. A trichogen cell was not associated with the peg organ.

The delicate thin walls, the spacious cavity filled with the vitreous substance and the elongated sense cells closely resemble the peg organs regarded by Wigglesworth (1939) as chemo-receptors. Since its chemical sense is poorly developed, one would not expect the mite to be well equipped with chemo-receptors. On the other hand, its perception of high or low temperatures is quite pronounced, and although the sensitivity to temperature differences is probably distributed over the body of the mite, a possibility is that the pointed peg organs may be temperature receptors.

Minute sensory rods. These minute sensory organs are about 6 μ long and 0·5 μ in diameter (see Fig. 22E). They arise from a chamber comparable in size to that of the large peg organ. It was extremely difficult to be certain whether or not the end-organ is thin walled. However, there is no sign of a sensory nerve enclosed by thick walls. They are principally confined to the first leg. There is one on each of the three distal segments, and one is also present on the tarsus of the second leg. In some insects such rods are grouped inside sunken epidermal pits and they have an olfactory function.

Claws. The trifurcate claw, at the end of each tarsus, consists of a basal part and three very curved distal branches, the middle one being the longest. The proximal part of each branch is dilated (see Fig. 22E). In section each branch is shown to be thick walled and supplied with a sensory nerve extending to the tip. Within the base of the claw the sensory nerves to the three branches are separate, and each exhibits a dilatation before joining the main nerve of the leg (see Fig. 22C).

Sense organs on the palps

Most of the sensilla are confined to the papilliform tarsus. Two peg organs are present, one with a round tip and the other with a pointed one (see Fig. 22A). The concentration of both tactile and chemo-receptor organs on the tarsus (see Fig. 23C) is to be expected, since the palps play an important role in testing the skin before the digits of the chelicerae are buried in it.

Sense organs elsewhere on the body

Plumose setae. Both the dorsal and ventral surfaces of the body are covered with these sensilla. About thirty are arranged in transverse rows on the dorsal surface, excluding those on the scutum, and about the same number are similarly arranged on the ventral surface.

Pseudostigmatic setae. The so-called pseudostigmatic setae usually arise from a mid-position of the scutum. In related genera these organs are club-shaped and closely resemble the similarly termed organs, typical of members of the Oribatidae.

The club-shaped organs of orbatid mites by the nature of their structure were thought by Michael (1883) to be connected with hearing or smell, and he inclined to the former.

The pseudostigmatic organs of *T. autumnalis* bear no resemblance to the club-shaped organs mentioned. They are, however, quite distinct from the rest of the sensilla so far described. The hair itself is about 70 μ long, very slender, curved and equipped with barbs on the distal portion. The hair arises from a relatively large basal process which fits into the space of the cuticular covering like the ball part of a ball-and-socket joint. In section the basal process is seen to have a spacious chamber about 8 μ in diameter, which is enclosed by the convex dorsal surface and a pronounced concave ventral surface. The inside wall of the chamber is distinctly ridged (see Fig. 23 D). The significant feature is that this basal process, which in

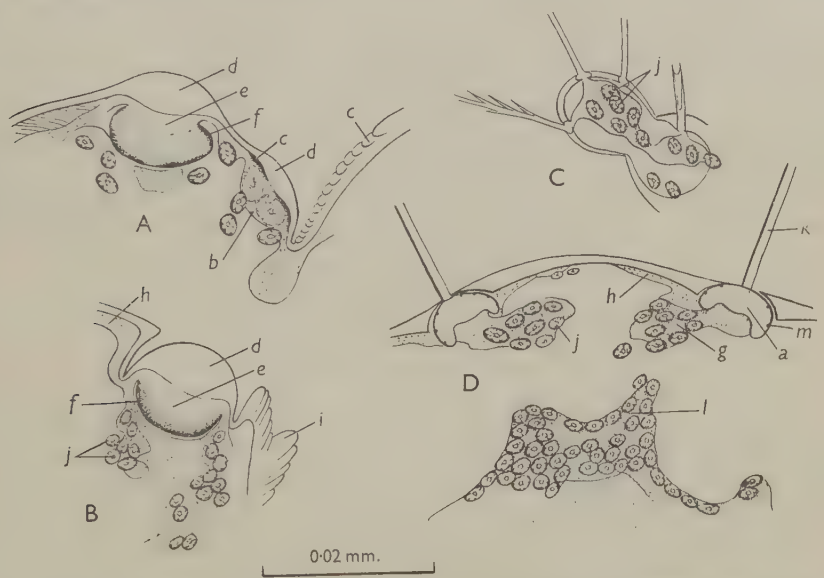


Fig. 23. A, sagittal section of photoreceptor through anterior and posterior eyes; B, transverse section of anterior eye; C, horizontal section of right palp; D, transverse section of 'pseudostigmatic' organs. *a*, basal part of 'pseudostigmatic' organ; *b*, cells below lens of posterior eye; *c*, black pigment; *d*, lens; *e*, red pigmented oily substance; *f*, pigment cup; *g*, sense cells; *h*, hypodermis; *i*, cuticle; *j*, nuclei of sense cells; *k*, 'pseudostigmatic' hair; *l*, outer cortex of supra-oesophageal ganglion; *m*, ridge.

transverse section looks like an inverted cup, would appear to be capable of a rotating movement within the socket. Furthermore, this basal process projects into the body cavity. Below each basal process is an aggregation of nerve cells with a thick strand extending to the ventral concave wall. These aggregations of nerve cells are quite close to the dorsally extended parts of the supra-oesophageal ganglia (see Fig. 23 D).

The end-organ is not thin walled, and thus it would seem that when it touches some object the pressure exerted may cause a rotatory movement of the basal process. These stiff setae in their natural position extend well above the other dorsal setae of the body (see Fig. 14). One would therefore expect them to have a sensory perception of touch and probably of vibrations in the air.

The ocular areas

The ocular areas lie on each side of the scutum near the postero-lateral border. In the live mite they appear as two conspicuously red oval patches, but each area is composed of two well-defined eyes, an anterior and a posterior one.

In sagittal section the two distinct eye structures are clearly visible. The cuticle of the anterior one is thickened into a distinct elliptical lens. Below the lens is a well-defined discoid mass of red-pigmented oily substance surrounded by a pronounced dark-coloured, almost black, pigmented cup. The posterior eye is not so well developed. The lens-like cuticle is only about twice the thickness of the normal cuticular covering and half that of the lens of the anterior eye. In a transverse section of the anterior eye, the discoid mass of red-pigmented substance is continuous on both sides, with laterally placed nerve cells (see Fig. 23 B).

It is noticeable that the anterior eye will be exposed to rays of light from many directions, but the posterior one is restricted in its range (see Fig. 23 A). In the case of the better developed anterior eye one may assume that the thickened cuticular covering, acting as a lens, brings the longer light rays to focus on the wall of the pigment cup; and that the shorter light rays after being reflected from the inner surface of the cup are focused on the photosensitive substance.

IDENTIFICATION OF THE RESPONSES

The responses of the hexapod mite to various stimuli have been identified, where possible, according to the classification of Fraenkel & Gunn (1940), because the adoption of some recognized scheme is essential if the expression of results of work on the sensory physiology of invertebrates is to be consistent.

The peculiar questing response of the harvest mite to a sudden decrease of light intensity (shadow reflex), or to vibration, is, like that of the tick, a postural and not a locomotor activity. It cannot therefore be identified with either the random (kineses) or the directed locomotor reactions (taxes) which distinguish the two basic groups of the classification.

Random movements

A random movement or kinesis involves the stimulation of an animal into locomotor activity, but the body axis of the animal shows no orientation towards or away from the stimulus.

Observations in the field showed that mites of a cluster, after questing in response to shading or vibrations, invariably moved away from the site of the cluster. The questing response of the mite is a prelude to locomotion, and in this respect it closely resembles the behaviour of the tick (Lees, 1948). The forward movement resulting from the stimulatory effect of shading or vibrations is an example of orthokinesis. The activity of desiccated mites in a linear humidity gradient is typical of orthokinesis, the undirected movements eventually leading the mites into the moist region where movement is arrested. The response may also be defined as positive hygrokinesis.

The extent of stimulation by contact with each others bodies leads to low activity, a quiescent state being typical of mites in a cluster. When only the tarsi of mites touch a surface it leads to a high state of activity. On the surface of a collecting jar, or that of the human skin, the intensity of contact stimulation is low because only the tarsi make contact with the surface. The result is a high state of locomotor activity by the mite. This type of tactile response is an example of a low thigmokinesis, because a low intensity of stimulation by contact leads to a high state of activity. The high extent of contact stimulation experienced by the mite pressed between the skin and tight clothing of a human host reduces its locomotor activity. The mite is then induced to plunge the digits of the chelicerae into the skin, thus areas of the body where clothing is tight are typical sites of attack.

The activity of the mite moving at random over the skin of a young live mouse could be interpreted as an example of klinokinesis in response to the warmth of the body. On one occasion a mite traced a much convoluted path in response to two opposing beams of different light intensity (see Fig. 3). But a response of this type was not observed to chemical stimuli or temperature.

Direct movements

Unlike the random movement of the kinesis, a direct reaction or taxis involves the long axis of the body of the animal being orientated in line with a single source of stimulation. A positive or negative response depends on the mite moving towards or away from the stimulus.

The photopositive mite will move along the bisector of two equal intersecting lights before curving towards one of the sources, and when one eyespot is blinded it will make circus movements. Such responses are regarded as denoting phototropotaxis. When the mite is placed in a weak light the tracks at a distance from the source stimulate klinotactic behaviour (see Fig. 1 A). As the mite approaches the source the tracks straighten and tropotaxis is well defined. The reaction is more pronounced in strong light and is also evident in the orientation of the mite towards a laterally presented beam of light. The response to reflected light is an example of skototaxis.

The mite is unable to locate a warmed glass tube or the body of a young live mouse. The avoiding responses to volatile substances, perspiration, high humidity, the darkened portion of a linear light-intensity gradient, and low and high temperatures outside a preferred zone, are all examples of negative responses to various stimuli.

THE SIGNIFICANCE OF THE RESPONSES IN THE NATURAL ENVIRONMENT WITH RESPECT TO ACQUIRING A HOST

The analysis of the sensory perceptions of the mite suggests that its behaviour in the natural environment is a series of simple responses to stimuli which form the complex pattern of the micro-habitat. It is clear, however, that the intensity of response to different stimuli varies, the response to mammal skin, for example, dominating the positive response to light when the combined stimuli are offered.

One would expect the behaviour of a potential parasite living freely on the soil to be designed for acquiring a host, and hence that the stimuli encountered by the unfed

mite in the natural environment would be of value in this respect. When dry conditions prevail the locomotor activity of the mites over the surface of the soil or low-lying vegetation is almost exploratory in character, but there is nothing to suggest that this behaviour is directed towards seeking a host as suggested by some observers. In describing the movements as representing a trial and error method it would be reasonable to infer the activity of the mite to be a series of avoiding reactions, the mite being led into more favourable situations by moving away from those which induce a negative response. This behaviour would explain the restriction of mites to the moister pockets of the macro-habitat.

The mites are unable to withstand prolonged exposure to low humidities and are thus confined to moist habitats. This is due to the high permeability of the cuticle to water, since mites soon shrivel up if kept in a dry Petri dish exposed to the heat of strong sunlight. Depletion of the normal water content stimulates the mite to activity in the natural environment, locomotor activity being maintained until the mite enters by chance a moister part where the rate of movement is slowed down. When the macro-climate is dry desiccation certainly prevents the mites from climbing upwards very far from the soil. Should they climb a twig or dead leaf they encounter dry air which induces a high locomotor activity, and this will lead them back to the moist soil to replenish any loss of water by evaporation. It is significant that mites are difficult to locate when the soil is dry.

We may therefore deduce that the chances of a mite encountering a host are considerably lessened in dry conditions. But when climatic conditions are favourable, for example, overnight rain followed by spells of warm sunshine, the combined stimuli of warmth, fluctuating light and high humidity induce the mites in the micro-habitat to form clusters. The warmth stimulates the mites into activity, the sunlight induces the upward-climbing response, and the high humidity of the air allows the mites to reach the uppermost parts of the soil, dead leaves, twigs and low-lying vegetation without losing water by evaporation. The mites are drawn up from the soil, but a single mite will seldom remain alone upon the uppermost point of its immediate surroundings. It will climb down and then up to the tip of an adjacent rise in the soil, a twig or dead leaf. Mites of a concentrated pocket will, however, usually encounter each other at high points of the micro-habitat. On touching each other they become still and form a quiescent cluster. Individual mites join up at the fringe until the cluster in parts may be formed of two or three layers of mites. It was noticeable that warm spells of sunshine, provided the humidity of the air was favourable, invariably induced clustering. The aggregated mites usually rested upon a lump of soil, a twig, or a leaf in a direct line with the sun, which often meant that clusters were not always formed upon the uppermost points available on the material supporting them. Such behaviour is readily demonstrated in the laboratory by mites transferred to a piece of crumpled filter-paper illuminated from the side by a lamp. The mites aggregate in positions, nearest to the source of light, irrespective of whether they are the highest points available. In the natural environment the mites showed a predilection for resting upon the sharp edges of the supporting material.

The formation of clusters at some distance above the soil would give the mite the

best opportunity of encountering a host. It is significant that clusters, once formed, will persist after dusk. The gradual decrease of light intensity has no influence upon the mites, since the high intensity of stimulation by contact with each other keeps the cluster intact. This persistence after dusk synchronizes with the movements of rabbits, field mice, bank voles and other common hosts mainly nocturnal in their habits. During daytime, birds, domestic animals and some wild animals are the potential hosts. But the mites on attaining a favourable position must still depend on the chance approach of a suitable host.

The response either to shading or to vibrations is clearly one of preparedness. A potential host casts a shadow across a cluster of mites, and a quiescent cluster would be transformed into a mass of questing appendages. Shading will also stop a mite in its tracks; the body rests on the substratum and all the legs are directed upwards. The questing response of mites to vibrations may have great value at night when leaves and twigs, infested with clusters, are disturbed by nocturnal hosts.

Certain sensory perceptions which might be of value in locating a warm-blooded host are not possessed by the mite. It is incapable of orientating towards a host by detecting the warmth or odour emanating from the body; but on touching a moving object, inanimate or otherwise, the mite displays a remarkable ability for clinging to it. If the mite touches the skin it shows a well-defined response to body heat as shown by tests with a live mouse. The intensity of the response to the skin of the host is so strong that the natural positive response of the mite to light is submerged, the avoiding reaction to darkness entirely disappearing. Hence the readiness of the mite to move unhesitatingly to the covered parts of the human body. The role of the olfactory sense probably becomes more evident on close contact with the skin, and the negative response to perspiration, varying in intensity according to the host, partly explains the apparent preference by the mite for one person more than another.

On the host itself the mites maintain a high state of activity for a varying period of time. During this pre-attachment phase there is a heavy mortality rate owing to scratching by the host. Those mites survive which eventually reach and attach themselves to parts of the body where they escape the attentions of the host. Keay (1937) gives a list of habitats on both mammal and bird hosts: inside the ears, between the digits, and around the anus being typical sites. The ankles, groin, waist, axillae and neck are favoured regions of attack on the human body. These are areas where the clothing is usually more tightly pressed against the skin, and the mite in such areas, by moving in a restricted space, becomes still in response to a high intensity of stimulation by contact. The palps are applied to the surface of the skin, the body is tilted and the cheliceral digits are buried into the horny layer (Jones, 1950*b*).

The mite feeds for about 3 days before detaching itself. The engorged mite climbs over the surface of the body before eventually dropping to the soil. Its further existence depends entirely upon the moisture content of the soil. In the laboratory engorged mites transferred to the surface of soil penetrate downwards; thus it is conceivable that under favourable conditions little or no lateral movement on the

surface of the soil precedes this apparent photonegative and photopositive geotactic behaviour. The return to the cool soil from the warmth of the body is evidence of a complete reversal of behaviour when the physiological state of the mite has changed. This penetration down into the soil is most probably a response to humidity, because recently fed mites are photopositive (see Fig. 24).

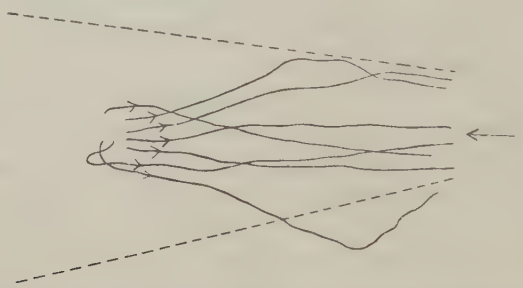


Fig. 24. Paths of fully engorged harvest mites, which had been attached to a host 6 hr. previously in a horizontal beam of light of 2600 m.c. (→) at the source.

Development to the nymphal stage will only take place under very moist conditions, and since the transition stage exists, for a period of about a month under natural conditions, as a form incapable of locomotion, it is understandable that deep penetration of the soil is essential to counteract desiccation. It is one of the striking features of the life cycle of *T. autumnalis* that the ectoparasitic larva is followed by a nymph and adult which lead a permanent hypogeal existence below the surface of the soil.

SUMMARY

Responses to stimuli

Light. In a strong beam of light the harvest mite will move directly towards the source, whereas in a weak light the tracks are at first inclined to be wavy, but as they approach the source the tracks straighten. The mite moves along the bisector of two intersecting lights of equal intensity, and when blinded on one side makes circus movements.

When offered a linear gradient of light intensity the mite avoids the darkened portion and moves towards the lightest part of the field. Its movement towards sunlight is a true response to light and not to heat. A sudden decrease of light intensity produces a questing response.

Temperature. The sensory perception of heat is poorly developed. The mite is incapable of locating a warm tube or the body of a young live mouse. On touching a heated object it displays a well-defined response to a temperature difference of about 15° C. In a linear or concentric temperature gradient it displays avoiding reactions to low and high temperatures and appears to prefer a range extending from 15 to 26° C.

Chemical stimulation. The mite is repelled at a distance of 0·5 cm. from phenol, methyl phthalate, dilute ammonia, xylene and a 3% solution of glacial acetic acid.

Toluene was repugnant at 1·5 cm., whilst a mixture of amyl acetate and water repelled the mite at 5 cm. Complete indifference was shown to the odour of skin, liver, sebum and cerumen, but perspiration induced an avoiding reaction.

Humidity. Depletion of the water content influences the response of the mite to humidity. A desiccated mite is active in dry air and inactive in moist air, but a normal individual will settle in either moist or dry air, while avoiding saturated air. The mite requires high humidities for prolonged survival, but avoids free water.

Touch. Unfed mites are very sensitive to touch. The extent of stimulation by contact with each other's bodies, which is regarded as high, immobilizes them, and it is primarily responsible for the quiescent state of a cluster of mites. When the stimulation is low, for example, when only the tarsi are in contact with a surface, the mite responds by displaying a high state of activity. A mite lightly touched will immediately quest, a response induced equally by vibrations of the substratum.

Clustering

The gregarious habit of the mites is primarily a response to the touch of each other's bodies. When the humidity is within the range 95–100% R.H. light will induce the mites to climb up a rod and form a cluster at the tip. Whether or not negative geotaxis also plays a part, it is difficult to say, because the evidence suggested that the mite is independent of gravity.

Sense organs

There are three types of sensilla: (1) tactile sensilla, both plumose and plain; (2) peg organs; (3) minute sensory rods, principally confined to the first leg. An elliptical lens, a discoid mass of red-pigmented oily substance, and a pronounced dark pigmented cup are conspicuous features of the better developed anterior eye of each ocular area.

Identification of the responses

Where possible the responses of the mite to various kinds of stimuli have been identified according to a recognized scheme of classification.

Behaviour in the natural environment

The responses to stimuli which the mite will encounter in the natural environment, and their value with respect to acquiring a host, are discussed.

I wish to thank Prof. Ritchie for his helpful criticism and interest, and to acknowledge the friendly discussions I had with the late Dr Gross on this work. Miss Catherine Hay kindly helped to prepare some of my sectioned material.

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